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ISOTOPES AND RADIATION IN PARASITOLOGY

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ISOTOPES AND RADIATION IN PARASITOLOGY

PROCEEDINGS OF THE RESEARCH CO-ORDINATION MEETING
ON THE USE OF ISOTOPES AND RADIATION
IN STUDY AND CONTROL OF PARASITIC DISEASES
ORGANIZED BY THE
JOINT FAO/IAEA DIVISION OF ATOMIC ENERGY
IN FOOD AND AGRICULTURE
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VIENNA, 1968

ISOTOPES AND RADIATION IN PARASITOLOGY
(Panel Proceedings Series)

ABSTRACT. Proceedings of the first Research Co-ordination Meeting of the joint FAO/IAEA Programme on the Use of Isotopes and Radiation in Studies on the Etiology, Effects and Control of Parasitic Diseases in Domestic Animals. The meeting was held in Vienna, 31 July - 4 August 1967, and was attended by the Programme's chief scientific investigators and several experts in parasitology who were not participating in the Programme itself.

The Contents includes, among others, papers on immunization experiments with irradiated larvae of *Syngamus trachea* in chickens, some practical aspects of the production of radiation vaccines against helminthic diseases, resistance produced in rats and mice by exposure to irradiated *Plasmodium berghei*, vaccination of cattle against trypanosomes by infection and treatment, immunological studies on *Fasciola hepatica*, and the anaemias associated with helminthic infections. The papers are provided with abstracts, with the exception of one short communication, and are followed by the recommendations of the meeting.

Entirely in English.

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FOREWORD

The application of ionizing radiation to produce vaccines against helminthic diseases was reviewed by an expert panel which met in Vienna in 1963 (IAEA Technical Report Series No. 32). This topic was again discussed at an advisory panel on the Use of Isotopes and Radiation in Animal Sciences in 1965. The prospects of using isotope tracers to study questions of the immunology and pathophysiology of helminthic and other parasitic diseases which could not easily be studied by other methods were also touched upon at the 1965 meeting and, since then, have been reviewed more extensively within the Animal Production and Health Section of the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture.

A co-ordinated research programme entitled "The Use of Isotopes and Radiation in Studies on the Etiology, Effects and Control of Parasitic Diseases in Domestic Animals" was initiated in 1966. As its title indicates, the programme considers all aspects of the applications of nuclear techniques to parasitology. Ten scientific institutes in nine Member States of the Food and Agriculture Organization of the United Nations and/or the International Atomic Energy Agency are at present taking active part, namely, one each in the Czechoslovak Socialist Republic, Denmark, Hungary, Israel, Italy, Kenya, Yugoslavia and the United States of America, and two in the United Kingdom.

Although the programme was limited from the beginning to parasites in domestic animals, it soon became apparent that the similarity of certain parasitic infections in man and animals, the existence of cross infections, etc., would make it extremely difficult to keep any strict and unnatural species barriers. The limitation to a certain, in itself very heterogenous, group of domestic animals would to a great extent prevent progress in the area under investigation. Thus the main aims of the programme were to contribute to the control and subsequent eradication of major parasites of farm animals, and it was necessary to consider within it such work on other species that is of direct relevance to the problem in farm animals. The apparent similarity between human and animal trematode parasites, between, for example, *Trypanosoma*, *Theileria* and *Plasmodium*, clearly justifies the discussion of many of these diseases together.

The chief scientific investigators met in Vienna from 31 July to 4 August 1967 at the first Research Co-ordination Meeting. Several experts in parasitology who are not participating in the programme itself also attended, so as to broaden the basis of the discussions. The meeting thus served two purposes, i.e. to co-ordinate work within the programme and to constitute an expert panel to advise on its further development.

Sixteen scientific papers, covering vaccine production by means of radiation attenuation, immunology and pathophysiology of parasitic diseases, including methodology, were presented. Although no results are as yet available on the radiation approach to trypanosomiasis it was felt that, to provide a background for discussion, there should be some contributions on immunological aspects of trypanosomiasis in cattle, and two papers on this subject were included.

By publishing this volume it is hoped that encouragement will be given for further work, and information provided for all scientists interested in these subjects.

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IMMUNIZATION EXPERIMENTS WITH IRRADIATED LARVAE OF SYNGAMUS TRACHEA IN CHICKENS

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Abstract

IMMUNIZATION EXPERIMENTS WITH IRRADIATED LARVAE OF SYNGAMUS TRACHEA IN CHICKENS. Among parasitic nematodes experimentally treated so far by irradiation, Syngamus trachea appears to be one of the most sensitive species. To attain equivalent biological effects higher doses of gamma radiation are required compared with X-ray irradiation under the conditions described.

The pathogenic effects of the gapeworm being due as a rule to the attachment of worms in the trachea, irradiation of infective larvae for immunizing experiments should take place with doses which will reduce significantly the attachment of the larvae to the trachea. The author therefore used doses of 5 or 8 kR gamma radiation.

Preliminary trials were made with chickens one to three weeks old. The immunogen effect of irradiated larvae manifested itself in a reduced number (0 to 28%) of challenge worms compared with those of controls. While 24% of the immunized birds harboured worm pairs in smaller numbers, no worms could be detected at all in 76% of the birds. A more effective immunity resulted in groups of birds treated twice.

Keeping in view the exigencies under field conditions, the author attempted to extend investigations to day-old chickens also. As with pheasants and turkeys, though to a somewhat lesser degree, these can also be immunized. The immunity proved effective following heavy challenge infestations which caused the death of most of the controls. Development of immunity can be demonstrated as early as the sixth day following treatment; it attains a satisfactory degree on the tenth to twelfth day, and continues on into an age resistance.

To ascertain whether a single immunization of young chickens, when treated before their first feeding, was likely to protect them against natural infestation, attempts were also made to treat day-old chickens under farming conditions. Though exact investigations in this line present various difficulties with regard to the sometimes sporadic occurrence of syngamosis, yet results of the majority of the trials appear encouraging. Nevertheless, opinion as to the possibility of active immunization against the disease proving to be a valuable method of control requires further experimental work.

At the meeting of the Panel on the Production and Utilization of Radiation Vaccines Against Helminthic Diseases organized by the International Atomic Energy Agency in 1963, reports were presented on two marketed vaccines (Dictol, Difil) as well as on successful immunizing attempts against ancylostomatosis and schistosomatosis. At that time it was to be expected that before long additional information would be available on similarly produced vaccines against other parasitic diseases. Although irradiation has proved to be the method of choice for producing antiparasitic vaccines, their use and potency appeared to vary with the species of the parasite and host, as learned from various experiments in connection with schistosomatosis as well as from a number of more or less unsuccesful attempts to vaccinate against haemonchosis, ascaridosis, etc. Owing to the dissimilar immunological properties of the various parasitic diseases, optimal radiation dose and basic conditions of immunization should be established individually with each disease and species. In the present report are summarized the results of experimental immunization against syngamosis (gapeworm disease) by means of a radiation vaccine. Some details of this work have already been published,Varga [1-3].

GAPEWORM DISEASE (SYNGAMOSIS) OR GAPES OF BIRDS

The disease is caused most frequently by the roundworm species Syngamus trachea. Adult males usually attain sizes of 2 to 6 mm, and females 15 to 20 mm. The sexes, when adhering to the tracheal mucosa, are as a rule permanently in copula, therefore resembling the letter Y in shape. The worm is widely spread and parasitizes more than 50 species of birds. The veterinary significance of syngamosis is in fact considerable owing to its prevalance in pheasants and partridges and, in developing countries where farming and housing conditions are poor, also among fowls and turkeys. The infection takes place as a rule by the birds picking up eggs containing infective larvae from the contaminated soil, sometimes also free larvae or, mostly, by their swallowing lumbricoids, molluscs, arthropodes, etc., known to serve as reservoirs or transport hosts. From the alimentary tract the larvae migrate by the blood-stream via liver and heart to the lungs, begin to copulate after passing two moults and invade the tracheal tube as worm pairs on the sixth to seventh day following infestation, finally adhering with their cephalic end to the mucosa, reaching maturity about two weeks later.

The outcome of the disease depends on the age of the bird, a narrow tracheal lumen being obstructed more easily, on the number of worms attached to the tracheal mucosa, on the density of their location and, last but not least, on the susceptibility of the host species. In enzootic areas, mortality is as a rule considerable – in chickens it may attain 30 to 40% György and Tőth [4] and in pheasantries even up to 74%,Gräfner et al.[5]. The number of worm pairs causing choking and death of birds is relative. While four to five worm pairs adhering to the mucosa at the same height may cause suffocation, birds showing an even stronger infestation may be free of symptoms if the worm burden is scattered. If grown turkeys rarely succumb to syngamosis, their trachea usually harbours several hundred worm pairs.

Turkeys, pheasants and partridges are susceptible to S. trachea infestation even during adult age. Domestic chickens are susceptible only while young, becoming resistant with progressing age. Olivier [6] has shown that, following preliminary infestation, chickens, turkeys and pheasants may become immune to later reinfections. This was also confirmed by Sichobalova [7] in relation to chickens.

PRODUCTION OF INFECTIVE MATERIAL AND IRRADIATION

Syngamus eggs were secured from the droppings of artificially-infected turkeys by a flotation technique. The eggs, after filtering and repeated cleaning processes, were embryonated in Petri dishes at 28°C under a column of 2 to 3 mm tap-water, with appropriate aeration. Embryonation took about 8 to 10 days. Infective larvae were irradiated and used for treatment immediately, then later after storage for several months at + 4 to 5°C.

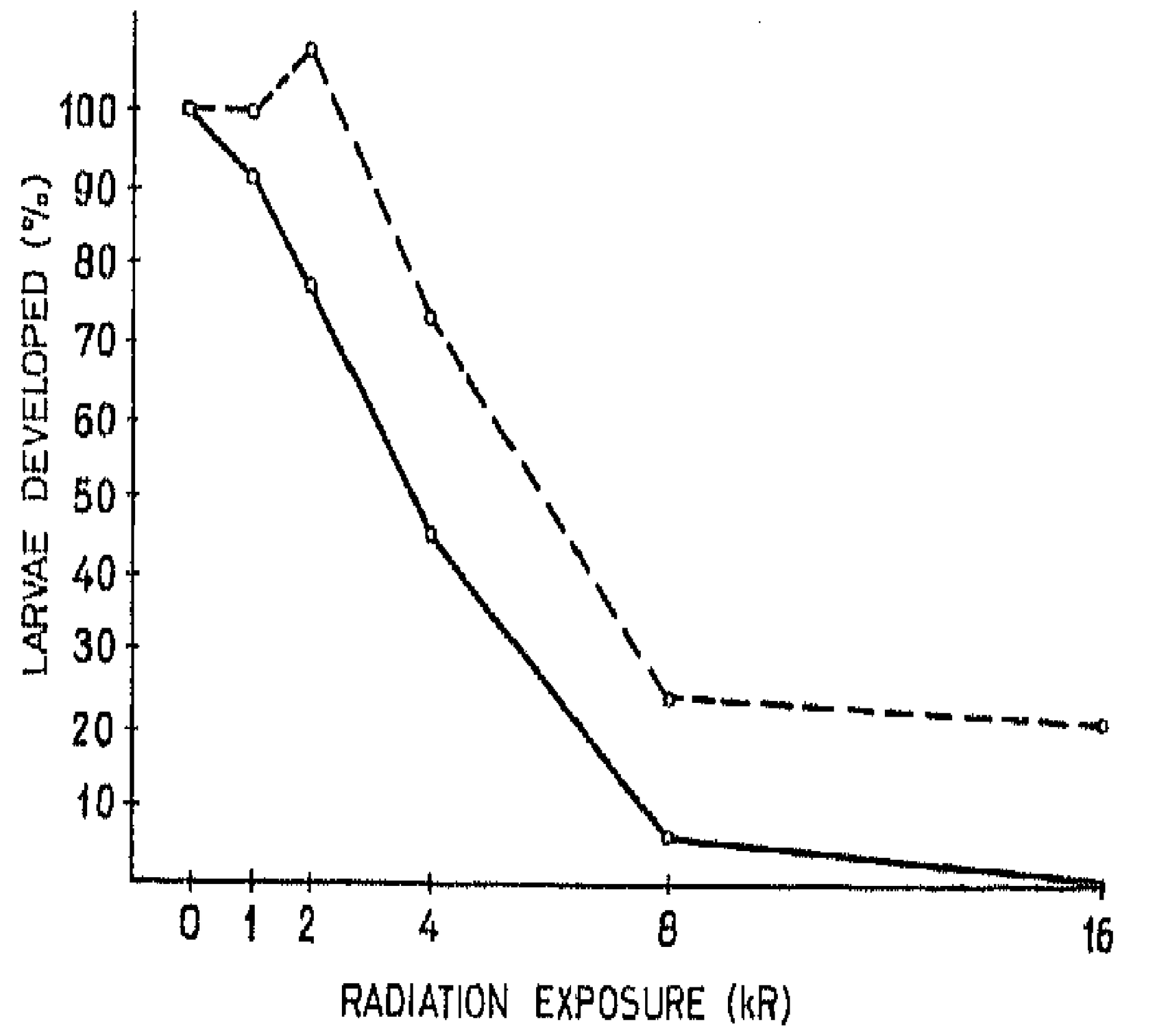

FIG.1. Percentage of larvae developed of S. trachea eggs irradiated with X-rays or gamma rays from ^{60}Co while in the fourth to eighth cleavage stage related to controls as 100.

——— X-radiation

------- gamma radiation

Eggs and larvae were usually treated by X-rays. For this purpose a Medicor THX 250-type therapy unit was used under the following conditions: kV 190, mA 15, filter 0, distance from water surface 20 cm.

In the past the air dose used varied between 573 and 1250 R/min. The total dose required was obtained by occasional calculation. Irradiation was performed at room temperature, usually continually. Larvae were suspended in tap-water to a density of 4000 to 40 000 larvae/ml. For irradiation with gamma rays from a ^{60}Co source they were placed in test tubes, and for X-irradiation they were placed in Petri dishes with a 2-mm-thick wall and internal diameter of 70 mm, standing on 3-mm-thick lead rubber. Calibration and periodic verification of the dose-rate were carried out by means of a Siemens universal dosimeter.

Occasionally a ^{60}Co irradiation unit with a 4 m water layer serving for protection and having a dose range of 103 to 270 R/min, was employed as the radiation source.

EFFECT OF IONIZING RADIATION ON EGGS AND LARVAE OF S. trachealis

The effect of radiation on eggs in the 4th to 8th blastomere stages was assessed by the number of developing larvae encountered after 14 days of incubation. Their counts were related to those obtained with non-irradiated eggs as 100. As shown in Fig.1, the retardation of larval development was directly related to the radiation dose. The difference between the effects of gamma and X-rays is explicable by the use of unfiltered X-rays of long wave-length exhibiting a superior biological activity compared to the more penetrating gamma rays. In fact, under the given conditions of experiment, an approximately 20% higher dose of gamma rays was needed to produce the same effect as X-rays.

The average figures for parasites in the lungs and tracheae of eight chickens per group infected when one day old with 400 irradiated third-

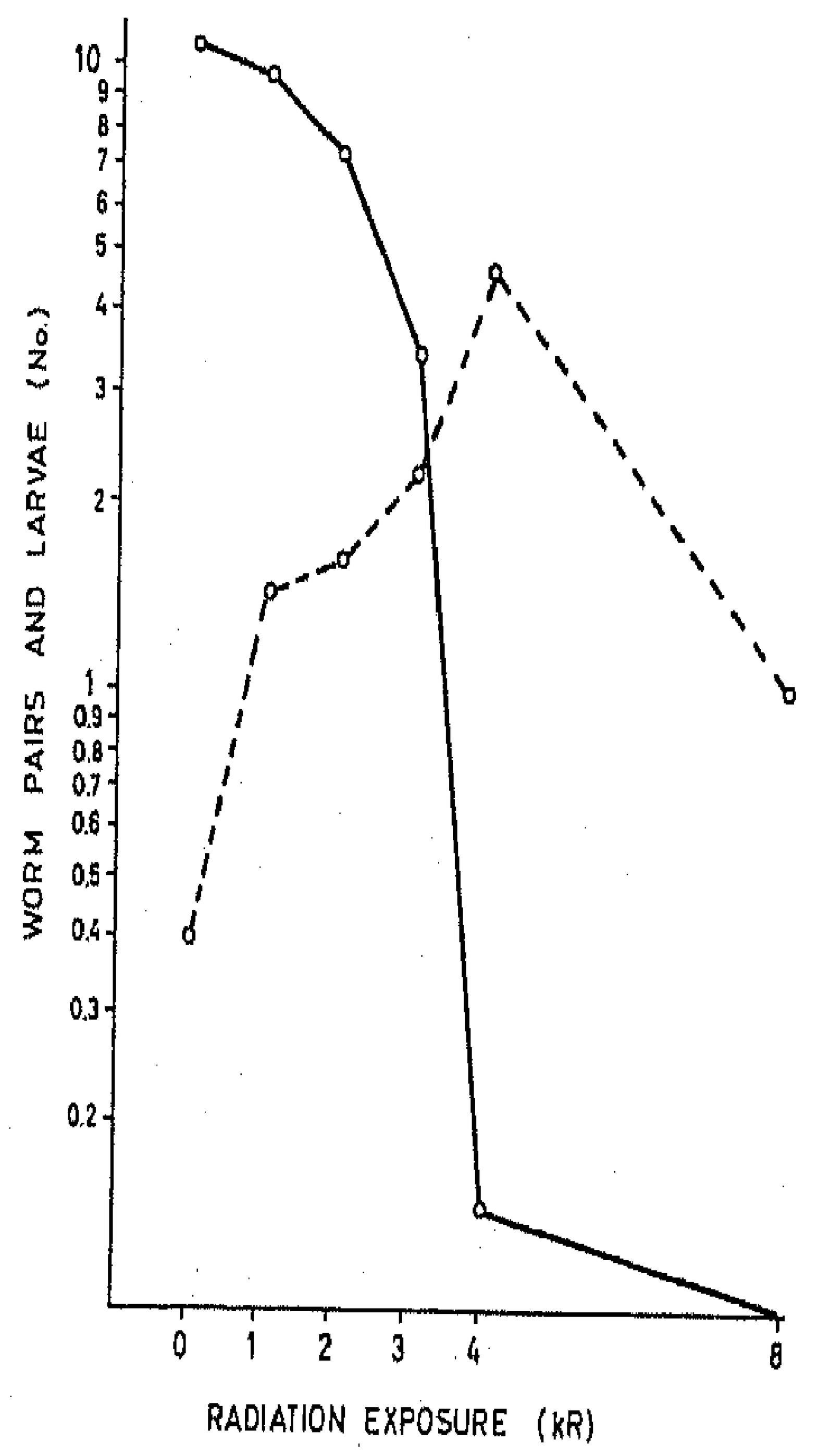

FIG. 2. Average counts per bird of worm pairs and larvae recovered from the tracheae and lungs, respectively, of chicks treated with normal larvae and larvae exposed to various X-ray doses.

——— worm pairs in tracheae
------- larvae in lungs

stage larvae per bird, and slaughtered between the 9th and 41st day of life, are shown in Fig. 2. At levels of 4 and 8 kR few and no worms, respectively, were found in the trachea, whereas in the lungs a fair number of larvae was still demonstrable. Adults developing from 1 to 3 kR-irradiated larvae produced few, if any, eggs capable of normal development. The undeveloped state of the reproductive organs was particularly conspicuous in the females. Using ^{60}Co gamma rays doses of 8 to 10 kR were necessary to prevent the attachment of worm pairs to the trachea.

For vaccination experiments larvae exposed to 5 kR X-radiation were used. This dose, although enabling partial development of larvae in the host organism, has been shown to reduce their attachment to the trachea sufficiently, as well as their pathogenicity.

IMMUNIZATION EXPERIMENTS ON CHICKENS ONE TO THREE WEEKS OLD

In most cases New Hampshire chickens were used. Preliminary experiments were performed to clarify whether or not Syngamus larvae attenuated by irradiation are capable of protecting the host against challenge. At two weeks of age firstly 800, then one week later 1200 irradiated larvae were administered into the crop with a pipette. After a further two weeks a challenge dose of 1500 normal larvae per bird

TABLE I. DATA ON CHICKENS IMMUNIZED [a)]

Group	Chicks (No.)	Worm pairs													Protection (%)
		per bird												Average	
I	12	2	0	0	0	0	0	0	0	0	0	0	0	0.16	98
Ic	12	18	15	13	10	9	8	8	5	5	4	3	3	8.41	-
II	11	3	2	1	0		0	0	0	0	0	0	0	0.54	98
IIc	11	67	50	47	35		31	30	13	9	7	6	5	27.22	-
III	12	1	0	0	0	0	0	0	0	0	0	0	0	0.08	98
IIIc	11	9	7	6	5	5	4	4	3	2	1	1		4.27	-

a) Immunized at the age of two and three weeks, challenged at the age of five weeks, and killed at the age of seven weeks.

was given to experimental and control groups alike. A particularity of syngamosis is that from the 8th day on the presence and burden of worms can be estimated by direct examination of the trachea.

The immune state of the birds was assessed by the comparison of the figures of worms encountered in the trachea of immunized and control chickens killed two weeks following the challenge, or spontaneously dying from the 10th day onwards. The particular importance of the count of worm pairs is inherent in the nature of syngamosis. The degree of protection was expressed in per cent terms, relating the number of undeveloped worm pairs to those found in the control groups as 100.

The data of the first experiment, the reproduced experiment and of a third experiment where challenge was made by feeding infected earthworms, are shown in Table I.

These and other studies indicate good immunogenicity of irradiated larvae, although in cull chickens, as well as with a single immunization, the rate of protection did not exceed 70 to 80%. Out of the chickens immunized once or twice when 1 to 3 weeks old, 76% were fully immune to challenge; also, the remaining 24% exhibited partial immunity, showing a worm establishment two thirds less than that in controls.

IMMUNIZATION OF DAY-OLD CHICKS

It appeared worth while to extend experiments to day-old chicks firstly, because baby chicks are known to have less immunological competence, and secondly, because syngamosis is a disease of young birds.

The data on chickens immunized with 800 larvae each when 1 and 4 days old, challenged when 14 days old and killed two weeks after challenge, are shown in Table II. About half the controls died of syngamosis between the 11th and 14th day.

TABLE II. DATA ON CHICKENS IMMUNIZED [a]

Group	Chicks (No.)			Worm pairs		Protection (%)
	Total	Infected	Negative	Total	Average	
I	13	4	9	14	1.67	94
C	12	12	0	224	18.66	-

[a] Immunized when one day old, challenged at the age of two weeks and sectioned after a further two weeks.

TABLE III. COMPARISON OF IMMUNITY RESULTING AFTER IMMUNIZATION OF CHICKENS [a]

Immunized at the age of	Chicks (No.)			Worm pairs		Protection (%)
	Total	Infected	Negative	Total	Average	
1 day	9	2	7	3	0.33	98
Control	7	7	0	119	17.00	-
2 weeks	12	5	7	50	4.16	42
Control	12	12	0	87	7.25	-

[a] Immunized with 500 irradiated larvae when one day and two weeks old, respectively.

Later on it was found that this immunizing dose can and must be reduced to prevent the development of lung lesions. A dose of 500 larvae produced less degree of immunity in chickens two weeks old than in those a day old (Table III).

One group of chickens, immunized when one day old and challenged two weeks later with 2000 larvae, was killed four instead of two weeks after challenge. In the immunized group all chicks survived challenge, while in the control group 8 of 12 chicks died of syngamosis. This indicates that the dose applied produced firm immunity.

Chickens immunized when one day old and challenged afterwards at various intervals were examined to estimate the time required for the establishment of immunity as well as its duration.

As shown in Table IV, an immune response was already demonstrable on the sixth day, reaching a satisfactory degree by the twelfth day and turning later on to a very likely life-long age resistance, as shown also by the lower intensity of infection in the control group.

TABLE IV. RESULTS OBTAINED WITH CHICKENS IMMUNIZED WHEN ONE DAY OLD AND CHALLENGED AT DIFFERENT INTERVALS

Group	Challenge		Chicks (No.)			Worm pairs		Protection (%)
	at days	Dose (larvae)	Total	Infected	Negative	Total	Average	
I	6	600	12	9	3	154	12.83	58
Ic			12	12	0	368	30.66	-
II	8	700	12	10	2	107	8.91	44
			12	12	0	181	15.08	-
III	10	800	12	5	7	44	3.66	75
IIIc			12	12	0	174	14.50	-
IV	12	900	12	3	9	17	1.41	94
IVc			11	11	0	245	22.27	-
V	14	1000	12	4	8	19	1.58	89
Vc			12	10	2	166	13.33	-
VI	28	1200	11	3	8	18	1.63	85
VIc			10	10	0	111	11.10	-
VII	49	1200	11	0	11	0	0	100
VIIc			10	7	3	21	2.10	-
VIII	70	2000	11	0	11	0	0	100
VIIIc			10	6	4	11	1.10	-

The importance of the satisfactory attenuation of larvae has been indicated by an experiment where larvae irradiated at 5, 10 or 20 kR were applied in identical doses. The respective rates of protection were 93, 51 and 0%, the latter being obviously due to excess inactivation of the larvae.

Attempts to immunize day-old cull chicks have consistently failed, indicating that firm immunity can be established only in healthy hosts.

In some groups immunization was carried out with larvae exposed to 8 kR ^{60}Co gamma radiation. These larvae also conferred satisfactory protection which was, however, of a somewhat less degree than that obtained with larvae exposed to 5 kR X-radiation.

On storage for 1.5 yr at +4 to 5°C irradiated larvae failed to regenerate and maintained their immunogenic properties. About 30% of them lost viability during storage. Therefore, prior to the distribution of irradiated

TABLE V. IMMUNE STATE OF TURKEY POULTS SECTIONED WHEN FOUR WEEKS OLD a)

Immunized group	Turkeys (No.)			Worm pairs		Protection (%)
	Total	Infected	Negative	Total	Average	
Double c)	9	8	1	56	6.22	93
Single b)	7	6	1	218	31.15	47
Control	11	11	0	737	67.00	-

a) After single and double immunizing exposure at one day old and challenge at two weeks old.

b) Dose: 150 larvae per bird when one day old.

c) Dose: 150 and 300 larvae per bird when one and four days old. Challenge dose: 1000 larvae per bird.

TABLE VI. DATA ON CHICKENS EXPOSED TO NATURAL INFECTION FOR FOUR WEEKS

Group	Chicks (No.)			Worm pairs		Protection (%)
	Total	Infected	Negative	Total	Average	
I	28	4	24	4	0.14	93
Ic	27	25	2	55	2.08	-
II	37	7	30	9	0.24	88
IIc	40	32	8	76	1.90	-

vaccines the viability of larvae should be carefully checked and the dose adjusted accordingly.

IMMUNIZATION OF TURKEYS

Differences between the immune states of turkey poults immunized on one occasion when one day old or on two occasions when one and four days old, and challenged when two weeks old, are shown in Table V.

The increase in immunity with age was shown by the fact that among poults immunized on a single occasion when one day old the rates of protection were 56 and 91% at 4 and 8 weeks of age, respectively.

FIELD TRIALS

For evaluation the field trials carried out by the author have been classified into three different categories.

The first category included poultry groups immunized under laboratory conditions when one and four days old and afterwards placed in infected premises for a period of four weeks. Subsequently, the birds were killed and the results evaluated on the basis of post-mortem findings. Table VI shows that, although the intensity of infection had been low, firm immunity developed in accordance with the given circumstances.

The second and third categories included minor flocks owned by smallholders and reared in infected premises. These birds were immunized on the spot.

The second category consisted of chicken groups where infection was diagnosed by tracheal examination at eight weeks of age and the results were referred to appropriate controls. In 53 smallholder flocks encountered in three different villages a total of 513 day-old chickens were each treated with a single dose of 150 irradiated larvae and 606 chickens served as controls. The results of this study which, owing to field conditions, are rough estimates rather than precise data, are summarized in Table VII. The intensity of infection was relatively low in the control groups, but in the immunized groups it was consistently lower.

In the three categories of field trials also come the routinely immunized chicken groups which had no appropriate controls, yet had a history of maximum 80% yearly losses regularly occurring from syngamosis prior to immunization. With these groups comparison of pre- and post-immunization losses has been the sole basis of evaluation. The results are presented in Table VIII.

IMMUNIZATION OF PHEASANTS

Two thousand one-week-old pheasants were placed in an infected forest after 560 of them had been immunized with 150 irradiated larvae per bird. Within half a year 21 of the untreated pheasants died of syngamosis, while no losses occurred among the treated ones. Ten of the immunized birds were revaccinated at eight weeks of age and afterwards challenged simultaneously with the rest; 2.5 worm pairs became established in the twice-vaccinated group and 5.3 in the once-vaccinated group compared to an average of 12.3 in the control group. This corresponds to protection rates of 80 and 58%, respectively.

POSSIBLE VALUE OF VACCINATION IN THE CONTROL OF SYNGAMOSIS

Experiments conducted chiefly under laboratory conditions suggest that Syngamus larvae attenuated by irradiation are capable of conferring

TABLE VII. RATE OF CHICKENS IMMUNIZED[a)]

Group	Chicks (No.)	Infected (%)	Negative (%)
I	116	23	77
Ic	115	63	37
II	177	47	53
IIc	271	63	37
III	220	9	91
IIIc	220	31	69

a) Immunized at one day old and found infected by clinical examination at the age of eight weeks, in relation to controls.

TABLE VIII. MORTALITY RATE OF CHICKENS IMMUNIZED UNDER FIELD CONDITIONS

Group	Chicks (No.)	Mortality (%)
I	187	0
II	120	1
III	245	11
IV	490	3
V	354	0.3

immunity on chickens, turkeys and pheasants. The immunizing ability of irradiated Syngamus larvae has also been demonstrated by Ziegler [8]. Since, in chickens, syngamosis occurs almost invariably among very young birds several weeks old, attempts have been made to protect them at a very early age. In view of these circumstances vaccination should be carried out preferably at the day-old age, regardless of the fact that in early life immune capacity is lower. Chickens being of relatively low value in the category of "useful animals", their repeated immunization would be uneconomic. A single vaccination at the day-old age should be preferred also for technical reasons. Although by this method of immunization the protection is of a less degree than it would be on vaccination at two to three weeks of age, it is still sufficient to prevent lethal syngamosis in the majority of the vaccines. In chickens, known to develop resistance with age, early immunization helps to abbreviate the duration of the critical "susceptible period". This implication, derived in principle, has been supported by some – unfortunately not very exact – practical observations. A precise evaluation could be made only with poultry groups which, after two-weeks' maintenance in the laboratory, were placed in an infected environment for four weeks and subsequently killed. The post-mortem findings of these birds support, though not in a numerical sense, the favourable results found with irradiated vaccines in the laboratory. The low intensity of infection found both in immunized and control groups seems to be explicable among others also by the absence of a massive infection in the birds' first two weeks of life, which actually represent the most susceptible period. Therefore, the fact that, among chickens immunized under field conditions mortality from syngamosis has been relatively low in contrast to previous years, has been due not only to vaccination, but partly also to the two-week period of isolation during which immunity was allowed to develop. On the other hand, the fact that smallholders have frequently disregarded the requirement for two weeks' isolation has often interfered with the evaluation.

It is recommended that chickens be vaccinated after hatching, prior to the first feeding and reared in isolation, preferably for 12 to 14 days, as required for the establishment of immunity. Nevertheless, since syngamosis occurs mainly in smallholder flocks, the latter requirement is difficult to meet. Failure of vaccination is most frequently due to inadequate isolation. Another frequent source of error is that vaccination requiring individual handling of the birds is, as a rule, carried out by unprofessionals, resulting in uneven dosage.

The available experimental results serve as a theoretical basis for active immunization against syngamosis. A technical advantage is that the parasite eggs used for vaccine preparation are easily obtainable from adult turkey donors and larvae can be stored for more than one year. Nevertheless, further precise and extensive studies remain to be carried out to decide whether the control of syngamosis in infected foci is merely of theoretical value or is routinely practicable by this method.

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DEVELOPMENT OF AN X-IRRADIATED VACCINE FOR ANCYLOSTOMIASIS

Current progress

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Abstract

DEVELOPMENT OF AN X-IRRADIATED VACCINE FOR ANCYLOSTOMIASIS: CURRENT PROGRESS. An effective vaccine against the dog hookworm, *Ancylostoma caninum*, is described. It consists of larvae, attenuated by exposure to X-rays and shows the following properties: (a) the immune response to irradiated larvae is greater than to normal larvae; (b) immunity persists at an undiminished level for at least eight months (vaccination performed during the first three months of life); (c) single vaccination gives good protection but two doses of vaccine given one month apart to pups three to four months old is more effective; (d) vaccination is successful even when the first dose is given as early as 72 hours after birth. Double vaccination commencing at this time protects eight-week-old pups against potentially lethal challenge; (e) pre-natal infection of pups can be largely prevented by vaccination of bitches as early as possible in their lives (i.e. when bitches intended for breeding are themselves pups).

The significance of the results for the preparation of a similar vaccine against human hookworms is discussed.

In 1963 at the meeting of a previous expert panel, a preliminary report was given on the possibility of developing X-irradiated vaccines against hookworm disease [1]. Since that time considerable progress has been made and it is now anticipated that in the near future an X-irradiated vaccine will be in commercial use to protect dogs against hookworm disease caused by infection with *Ancylostoma caninum*. Preliminary experiments with the hookworms of man (*Ancylostoma duodenale* and *Necator americanus*) have also been completed and the way now seems clear for further work with these hookworms in the experimental subhuman primates (i.e. the apes) with the aim of investigating and evaluating X-irradiated larval vaccines against these two helminth infections in an experimental system and with a view to their eventual application to hookworm disease in man.

Several experiments to investigate the various aspects of the immune response of dogs after vaccination with X-irradiated *A. caninum* larvae have been reported [2 - 9]. A single vaccination [2] was less effective than two doses of vaccine given one month apart [3] to three- to four-month-old pups when measured by necropsy worm burdens from the challenge infection, although even the less efficacious single vaccination was sufficient to prevent morbidity and mortality of the vaccinated pups compared with the severely affected controls. Vaccination with X-irradiated larvae was more effective when the vaccine was administered by subcutaneous inoculation than when administered orally [4]. Subcutaneous vaccination with X-irradiated larvae was more efficient than oral or subcutaneous vaccination with normal infective larvae [5]. Attempted vaccination with normal larvae proved also to be an extremely hazardous procedure to the health and survival of the pups.

Immunity was shown to persist at an undiminished level and in the absence of re-infection for at least eight months [6]. This persisting immunity from vaccination given in the first three months of life was augmented by a further resistance which developed in unvaccinated and uninfected dogs and bitches and which was attributable to age per se [7]. Vaccination with irradiated larvae was successful even when the first dose of vaccine was given as early as 72 hours after birth [8]. After double vaccination commencing at this time, eight-week-old vaccinated pups were able to withstand a potentially lethal challenge infection. Even adult dogs were shown to benefit from vaccination although age per se conferred a considerable additional resistance to primary infection [7].

An interspecific immunity in dogs between *A. caninum* and *Ancylostoma brasiliense* was shown [9], in that dogs could be protected against a challenge infection of the latter species by vaccination with X-irradiated *A. caninum* larvae. The practical significance of this observation resides in the importance of creeping eruption or dermal larva migrans as a cause of debility in man following exposure to the larvae of *A. brasiliense* and in the possibility that vaccination of dogs with X-irradiated *A. caninum* would contribute to a reduction in the incidence of this condition in man by reducing the general level of *Ancylostoma brasiliense* infection in the dog population [1]. However, in animal hosts (i.e., dogs and cats) *A. brasiliense* was shown to be practically non-pathogenic [10].

Results in preparation, or reported in preliminary form elsewhere, have shown that pre-natal infection of new-born pups can be prevented to a considerable degree by vaccination of bitches as early as possible in their lives (i.e. when bitches intended for breeding are themselves pups). Vaccination of the young bitch reduces subsequent prenatal infection in her puppies by 80 to 90% compared to the level of prenatal infection in pups born to unvaccinated bitches. In dog hookworm enzootic areas prenatal infection and neonatal reinfection constitute the normal state of affairs. Before use of an irradiated vaccine, anthelmintic treatment would be advisable to effect removal of the prenatally and neonatally acquired worms. In these circumstances it has been shown that neither immune tolerance in the pups nor passive immunity congenitally conferred on the pups (i.e. passive immunity from the bitch, which at this stage in her life is almost certain to be solidly resistant to reinfection with hookworm) interferes with the efficacy of vaccination. In a series of eight experiments using more than 50 prenatally infected pups, and retaining litter mates as controls, the results showed that the prenatally infected pups were successfully vaccinated with protection in terms of challenge worm establishment of the order of 80% compared with the controls. The challenge infection proved to be an LD_{50} for the controls and the surviving controls were severely affected by acute ancylostomiasis while the vaccinates were completely unaffected by their small challenge burden of hookworms.

Recent work in East Africa, in which more than 300 monkeys of various groups were used, has shown that only the apes (specifically chimpanzee) are suitable alternative hosts for the hookworms of man. The way for further work in this field is now apparent and it is to be hoped that the recommendations of an earlier meeting [1] may now be reconsidered in the light of this recent information. The report of a World Health Organization-sponsored conference on ancylostomiasis [11] contains the recommendation that the investigation on the possibilities of developing X-irradiated vaccines for hookworm disease of man should be undertaken.

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VARIABLES IN THE RADIATION-ATTENUATION OF HELMINTH LARVAE: TEMPERATURE/OXYGEN EFFECTS

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Abstract

VARIABLES IN THE RADIATION-ATTENUATION OF HELMINTH LARVAE: TEMPERATURE/OXYGEN EFFECTS. Experiments in the irradiation of N. brasiliensis larvae at different temperatures have shown a marked decrease of radiosensitivity with increasing temperature, particularly in the range of 20 to 30°C. This is probably due to an oxygen effect, i.e. a more rapid reduction of the oxygen content of the suspension at higher temperatures as a result of the greater metabolic activity of the larvae. Experiments where the oxygen content of the suspension was analysed favour this theory. Further experiments are necessary to check if there is also a temperature effect per se, apart from the oxygen effect.

Irradiation experiments with larvae should always be performed with temperature control, preferably within the range of 15 to 20°C, where the temperature effect is apparently insignificant.

INTRODUCTION

The necessity for the further investigation of important variables in the radiation-attenuation of helminth larvae was emphasized in the report issued in 1964 by the International Atomic Energy Agency [1]. Using N. brasiliensis larvae, information has been obtained about the importance of dose-rate, larval concentration and quality of radiation on the attenuation process, Jennings, Mulligan and Urquhart [2], Kassai, FitzPatrick and Mulligan [3].

The present paper summarizes some recent findings on the importance of the temperature at which irradiation is carried out.

MATERIALS AND METHODS

These were as described previously, Jennings et al. [2], Kassai et al. [3].

RESULTS

The results of two experiments on the temperature effect are summarized in Tables I and II. In almost all cases groups of ten rats each were infected with the various larval preparations. Control larvae were always kept at the same temperature for the same time as those undergoing radiation treatment. The "relative take" of irradiated larvae was calculated as the percentage of that in the appropriate control group.

The results in Table I show clearly that between 21 and 25°C there is a significant decrease in radiosensitivity.

The most obvious explanation of this phenomenon is in terms of the well-known "oxygen effect". The larval suspension at the higher

TABLE I. WORMS RECOVERED FROM RATS (Nos)
(Ten days after infection with 1000 N. brasiliensis larvae treated with 30 kR at different temperatures)

	Control				Irradiated			
Temperature (°C)	15	21	25	30	15	21	25	30
Mean worm burden ± SD	330 ± 163	289 ± 162	287 ± 149	288 ± 110	88 ± 59	61 ± 30	254 ± 71	320 ± 114
Relative "take" (%)	100	100	100	100	26.6	21.1	88.5	111.1

TABLE II. WORMS RECOVERED FROM RATS (Nos)
(Ten days after infection with 1000 N. brasiliensis larvae irradiated at different temperatures)

	Control		Irradiated	
	A	B	A	B
Mean worm burden ± SD	455 ± 132	477 ± 152	183 ± 33	383 ± 62
Relative take	100	100	40	80.3

A: irradiated at 20°C (dissolved O_2 = 4.48 cm^3/l).
B: irradiated at 28°C (dissolved O_2 = 3.41 cm^3/l).

temperature will of course contain less oxygen for purely physical reasons, but further depletion is likely because of the probable increased metabolic activity of the larvae at the elevated temperature.

Table II shows the results of an experiment carried out at 20°C and 28°C in which the O_2 content of the suspensions was measured. Whether the increased "take" at 28°C can be explained solely in terms of the lower O_2 content of the suspension must await further experimentation.

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SOME PRACTICAL ASPECTS ON THE PRODUCTION OF RADIATION VACCINES AGAINST HELMINTHIC DISEASES

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Abstract

SOME PRACTICAL ASPECTS ON THE PRODUCTION OF RADIATION VACCINES AGAINST HELMINTHIC DISEASES. The mortality rate of *Dictyocaulus viviparus* larvae increased much more rapidly on storage at room temperature than at 4° C. After one week of storage at room temperature it increased by 20%; at five weeks it amounted to 60%, which is 40% more than was found in the larval suspension held in the refrigerator.

The invasiveness of *Dictyocaulus viviparus* larvae was also markedly affected by the temperature of storage. The larvae stored in the refrigerator (at +4° C) showed a significant decrease in invasiveness between the fourth and fifth weeks, whereas at room temperature the same decrease of invasiveness was observed between the second and fourth weeks.

The viability and invasiveness for guinea pigs of larvae irradiated with 40 kR, and stored for five weeks, did not differ substantially from that shown by unirradiated larvae of the same age. A decrease in invasiveness was observed only shortly after irradiation (1.5 hours after irradiation) as shown by a significant decrease in larval counts recovered from the guinea-pig lung three days after infection. At 24 hours after irradiation the invasiveness of the larvae reached the mean values obtained for infection with unirradiated larvae.

Different exposures (280 R/min and 795 R/min) and different filtrations (0.5 mm Cu and 2.5 mm Al) had no significant effects upon the development of *Dictyocaulus viviparus* larvae in the guinea pigs, provided the same total dose of radiation was employed.

A. DEPENDENCE OF VIABILITY AND INVASIVENESS OF *Dictyocaulus viviparus* LARVAE UPON TEMPERATURE OF STORAGE

A substantial factor in experimental studies on *Dictyocaulus viviparus* infection in cattle, as well as in the production of an irradiated vaccine, is a sufficient number of viable and infective larvae. The invasiveness of third-stage larvae is influenced by a number of factors during cultivation and storage. An important role is played, among others, by temperature, because *Dictyocaulus* larvae, which take in no food during their ectogenous phase of development, show increases in the metabolic activity rate and consequently, in the rate of consumption of their food reserves at higher temperatures.

Poynter and Terry [1] called attention to the necessity to check the viability of larvae in the course of the vaccine production process. They mention, however, that larvae which are living and mobile are not necessarily capable of invading the mammalian host. A suitable method for checking the invasiveness of larvae is the experimental oral infection of guinea pigs.

Methods

Infective larvae obtained from the faeces of experimentally infected bull calves were washed off from the cultures and six days later divided into two portions. The suspension contained 10 000 larvae/ml. One portion (group I) was stored in a refrigerator (at approximately 4°C), the other portion (group II) was held at room temperature (at approximately 20°C). The viability of larvae of both groups was checked on the day of the start of the experiment and afterwards at weekly intervals. The proportion of motile, coiled and dead larvae was estimated by counting 3 X 100 larvae and averaging the respective results. Viability was expressed by the number of motile and coiled larvae, the latter representing a resting stage. Every week five guinea pigs were each infected with 5000 larvae of group I and a further five guinea pigs were similarly infected with larvae of group II. Ten days later the animals were sacrificed as previously described by Tománek and Procházka [2] and the counts of larvae were averaged.

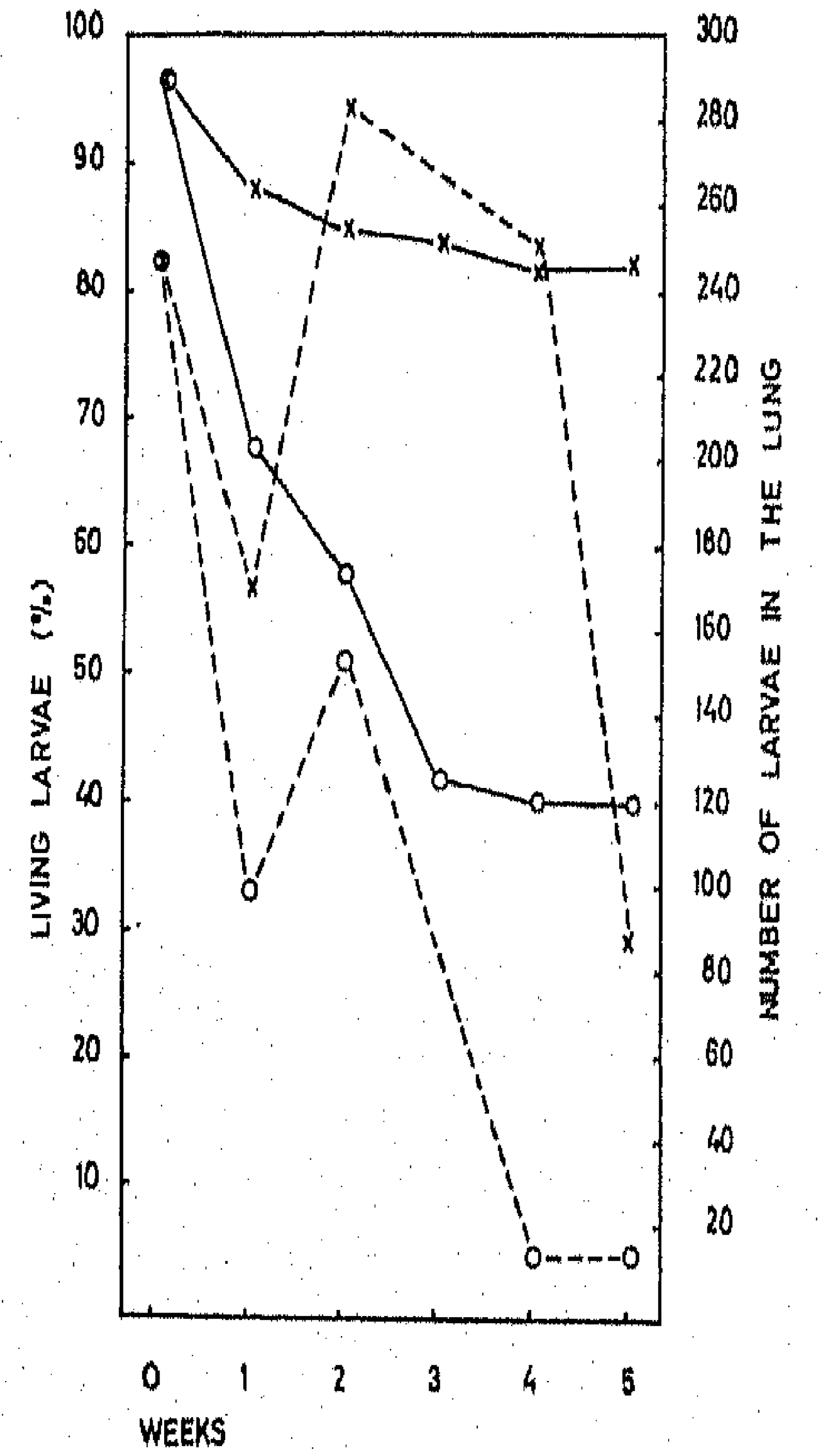

FIG.1. Viability and invasiveness of Dictyocaulus viviparus larvae.

——— per cent viability (motile and coiled larvae)

------- invasiveness expressed by the average count of larvae recovered from the guinea-pig lung 10 days after infection

xxx larvae held in the refrigerator

ooo larvae held at room temperature

Results

The initial viability of larvae employed for the experiment amounted to 97.3% (Fig. 1). After five weeks of storage in the refrigerator it descended to 82%, whereas in larvae held at room temperature it fell to 40%. The invasiveness of larvae of group I decreased significantly after four weeks. In the larval suspension held at room temperature there was a significant reduction in invasiveness between two and four weeks.

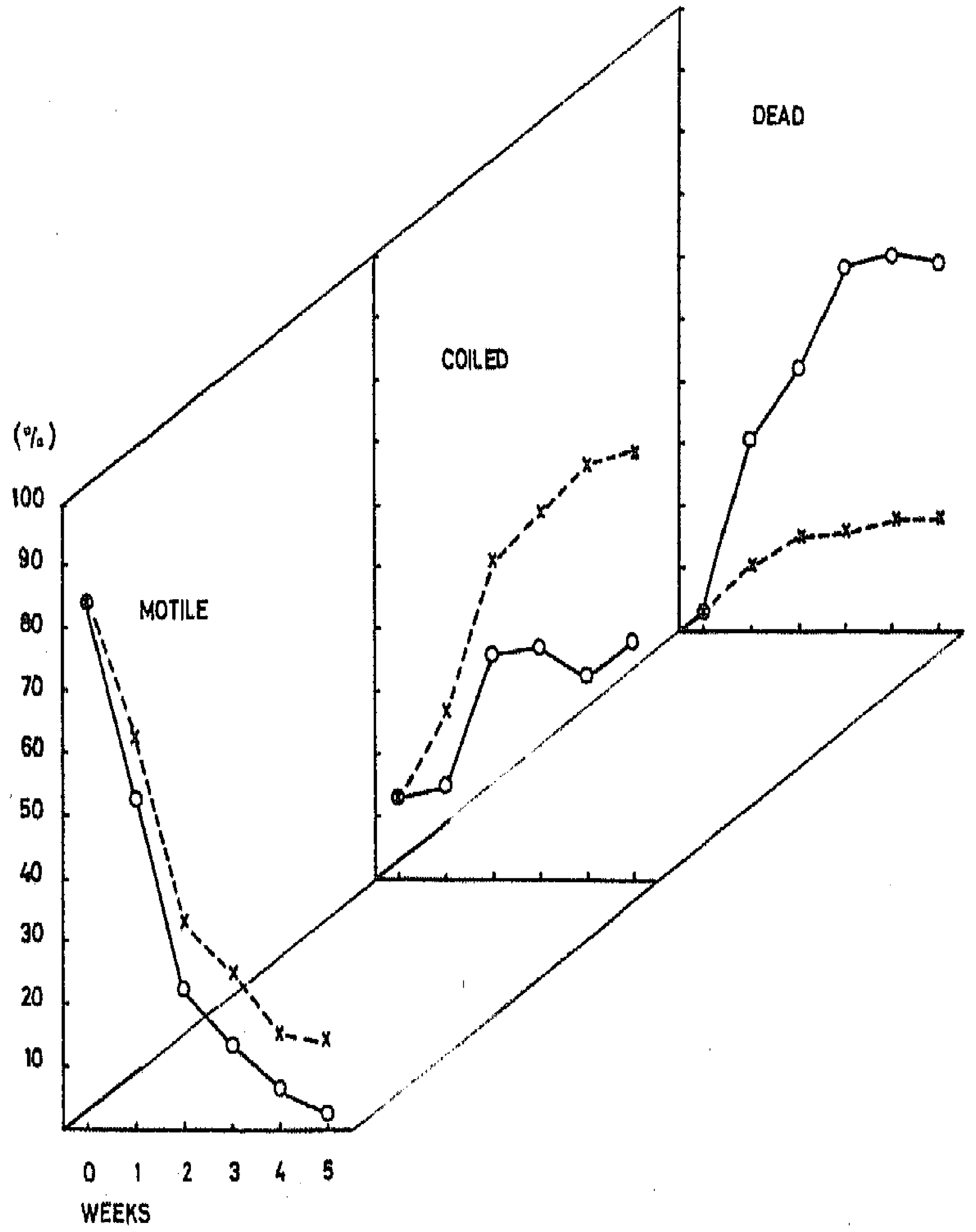

FIG. 2. Viability of Dictyocaulus viviparus infective larvae.

—X— larvae held in the refrigerator

—O— larvae held at room temperature

On storage at room temperature the mortality of larvae was 30% at one week and 60% at five weeks in comparison with mortality rates of 10 and 17%, respectively, shown by the larval suspension held in the refrigerator (Fig. 2).

Discussion and conclusions

Storage of larvae designed for the production of a vaccine is of considerable importance because Dictyocaulus viviparus larvae take in no food during their ectogenous development, and higher temperatures make them consume the food reserves (perceptible in the larval body as dark granules) more rapidly.

The mortality rate of larvae is markedly increased as early as one week after storage at room temperature, as demonstrated by the authors' experiment. The viability alone, however, is not the only decisive factor involved in the invasiveness of larvae. With an increasing period of storage the invasiveness of larvae, and consequently their immunizing capacity, decreases in spite of the same viability.

Storage of larvae in a refrigerator at 4°C reduces the mortality markedly but does not affect the invasiveness.

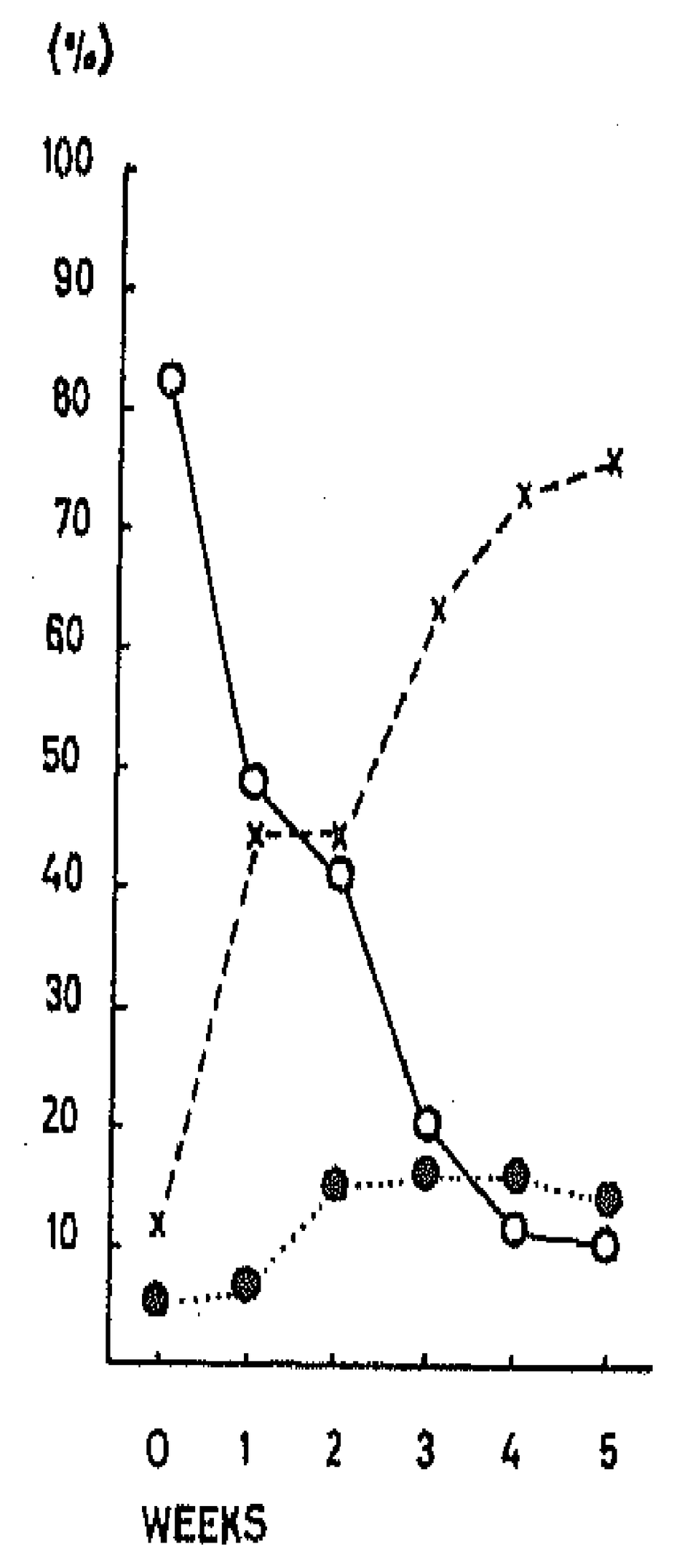

FIG. 3. Viability of *Dictyocaulus viviparus* larvae irradiated with 40 kR.

— O — motile larvae
--- X --- coiled larvae
··· ● ··· dead larvae

B. EFFECT OF LENGTH OF STORAGE UPON THE INVASIVENESS OF X-IRRADIATED *Dictyocaulus viviparus* LARVAE

To produce an effective irradiated vaccine against *Dictyocaulus viviparus* infection in cattle it is necessary to have living, partly attenuated larvae that would be capable of the same migration as non-irradiated larvae. Checks on invasiveness of irradiated larvae at varying times after exposure are an important factor in investigations of the shelf-life of the vaccine in view of the fact that during their ectogenous development the larvae carry out their metabolic activity only by utilizing food reserves in their own bodies. With the progressive consumption of these reserves the larvae gradually lose viability and invasiveness and eventually die.

So far, the mechanism of ionizing radiation effects on infective larvae is not well understood. Similarly, little attention has been paid to the relation between the invasiveness of irradiated larvae and the length of storage after exposure. The object of the present study was therefore to determine at what time after irradiation the mammalian host-invading capacity of irradiated larvae is reduced and whether it is substantially different from that shown by untreated larvae.

Methods

The infective larvae employed in this study were a pooled suspension of larvae obtained by washing off the coprocultures 2 to 12 days before irradiation. Prior to the experiment the suspension was held at 4°C. The larval suspension was then subjected to X-irradiation, as described

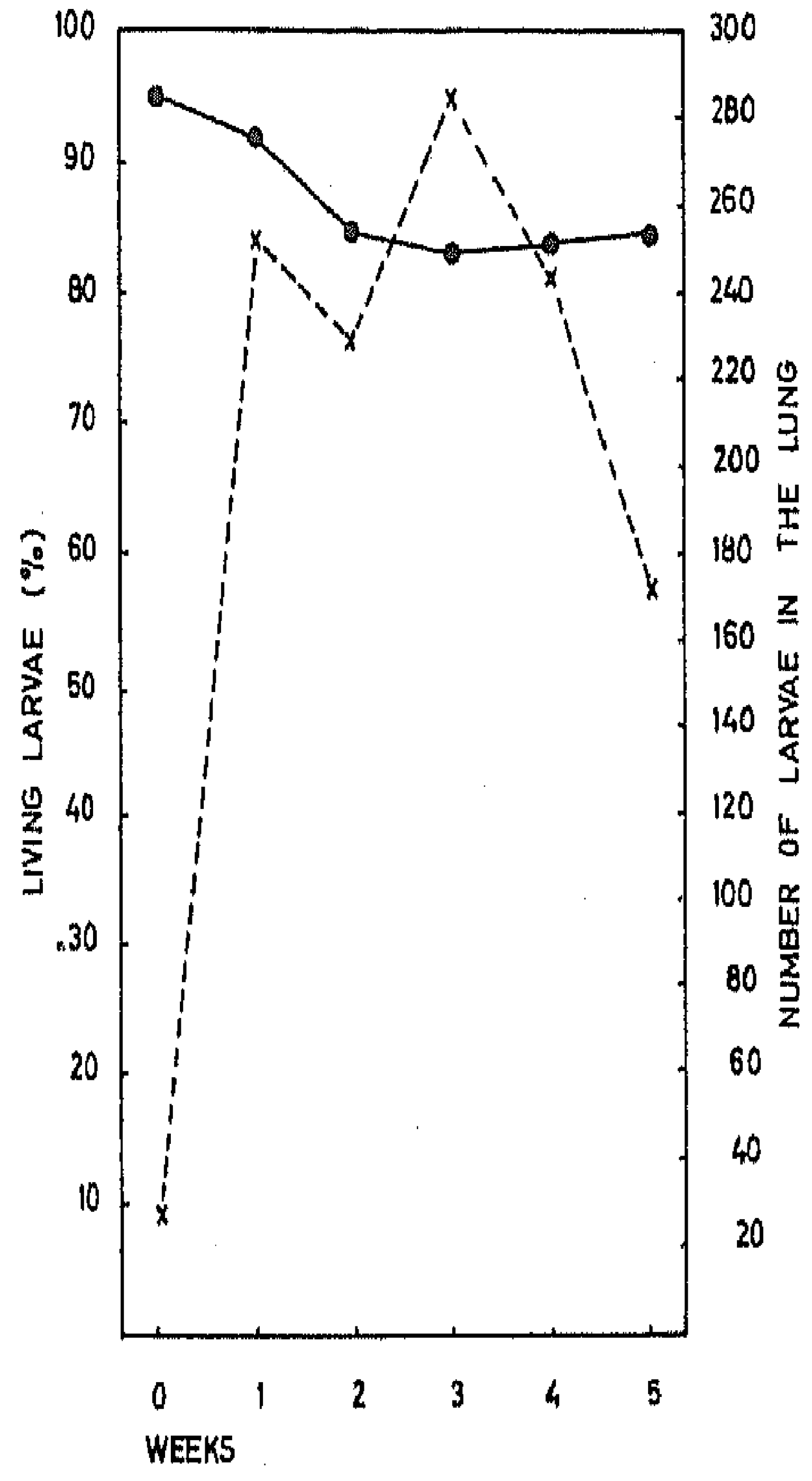

FIG. 4. Viability and invasiveness of larvae irradiated with 40 kR.

— ● — per cent viability (motile and coiled larvae)
--- X --- invasiveness of larvae expressed by the average count of larvae recovered from the guinea-pig lung at three days after infection

previously, Tománek and Procházka [2], the total dose of radiation being 40 kR. In the course of the experiment the viability of the irradiated larvae was followed. The invasiveness was tested in the groups of five guinea pigs infected with 5000 larvae each. Three days later the guinea pigs were killed, their lungs were examined for the presence of larvae and the average larval count was assessed. In the first experiment this examination was made at weekly intervals. In another experiment infection was made using larvae irradiated 1.5, 24, 72 and 168 hours previously. Throughout the experiment the larval suspension was held in a refrigerator at 4°C, which was found suitable in a previous study.

Results

As can be seen from Fig. 3, the irradiated larvae showed almost the same viability as normal untreated larvae of the same age (see Fig. 2). This is an important finding which confirms that the viability of larvae is not substantially reduced by irradiation.

In checks on the viability of larvae, the specimens recovered from the guinea-pig lung corresponded morphologically to the third stage but were exsheathed. It is of interest to note that the invasiveness is reduced shortly after irradiation as is evidenced by the fact that there was a significant increase in the counts of larvae recovered from the lungs of guinea pigs that were infected with larvae irradiated one week previously (Fig. 4). Thereafter, the average larval counts obtained continued

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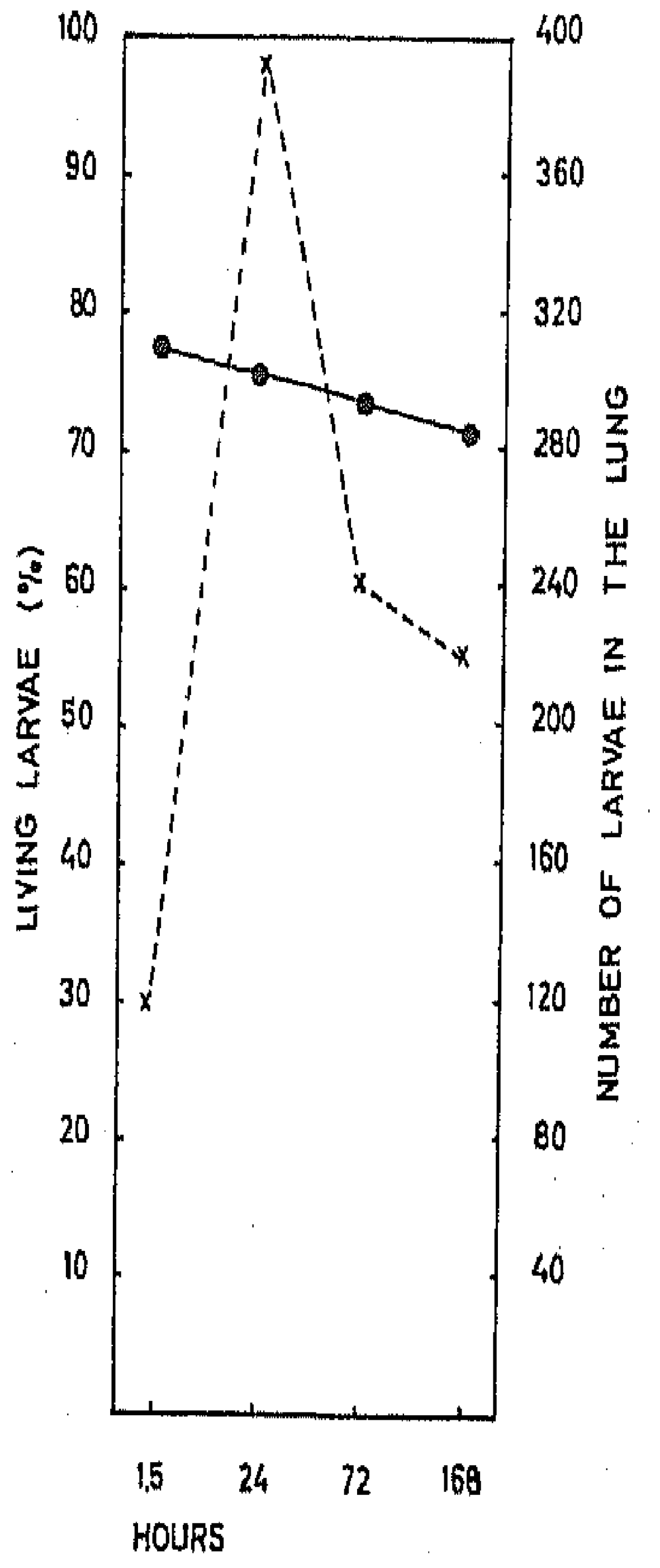

FIG. 5. Relation between the invasiveness of *Dictyocaulus viviparus* larvae irradiated with 40 kR and the length of storage.

—●— per cent viability (motile and coiled larvae)

--- X --- invasiveness of larvae expressed by the average count of larvae recovered from the guinea-pig lung at three days after infection

approximately at the same level and did not descend until the fifth week after irradiation.

Similar results were obtained in another experiment in which the relation between the invasiveness of irradiated larvae and the length of storage was studied at shorter intervals (Fig. 5). There was a highly significant increase in the larval counts obtained after infection performed at 24 hours after irradiation ($0.01 < P < 0.05$) in comparison to the larval counts recorded after infection performed at 1.5 hours after irradiation. When infection was made using larvae irradiated 72 hours previously the reduction in larval counts was at the border-line of significance in comparison to the preceding value ($0.05 < P < 0.025$), but there was a highly significant increase in larval counts in comparison with infection performed at 1.5 hours after irradiation ($0.02 < P < 0.01$). At 168 hours, i.e. at seven days, the increase in larval counts was insignificant in comparison with the initial value. The viability of larvae showed a slight progressive decrease throughout the experiment.

Discussion and conclusions

The comparison of results obtained with non-irradiated and irradiated infective larvae of *Dictyocaulus viviparus* stored at 4°C showed no substantial differences in the viability of the larvae. The invasiveness decreased at 1.5 hours after irradiation; at 24 hours after irradiation, however, it reached the mean values obtained after infection with non-irradiated larvae. In view of the fact that the capacity to reach the lungs is one of the decisive factors in immunization attempts with irradiated larvae, it is possible to employ this method as an indirect check on the shelf-life of the radiation vaccine. Viability checks alone were confirmed to be insufficient, in agreement with the data reported by Poynter and Terry [1]. The invasiveness of larvae recorded at various stages of the technological process may vary even under apparently identical technological conditions.

C. THE EFFECT OF DIFFERENT X-RAY DOSE RATES AND FILTRATION UPON INFECTIVE LARVAE OF *Dictyocaulus viviparus*

The technique of the irradiation of larvae is one of the important factors involved in the preparation of irradiated vaccines. Voltage, current intensity in mA, exposure (R/min), filtration and the total dose of radiation are mentioned by Mulligan [3] as decisive for the standardization of the technique. From the results obtained by Jennings et al. [4] on irradiation of *Nippostrongylus brasiliensis* larvae and by Urquhart et al. [5] on irradiation of infective larvae of *Haemonchus contortus*, it appears, however, that the dose-rate per minute has not a demonstrable influence upon the immunizing capacity of larvae, provided the total dose of radiation is the same. Likewise, Lucker and Vegors [6], who employed a Van de Graaf generator to irradiate *Dictyocaulus viviparus* larvae, believe that no fundamental role is played by the dose-rate per minute.

The effect of different X-ray qualities due to different filtration has thus far not been investigated in *Dictyocaulus viviparus* larvae. In the present study experiments were designed on guinea pigs to investigate the influence of different filtration and different X-ray dose-rates upon infective larvae of *Dictyocaulus viviparus*.

Methods

In the first experiment infective larvae of *Dictyocaulus viviparus* isolated from the faeces of experimentally infected bull calves were exposed to either 250 R/min or 500 R/min, by using a TUR 250 X-ray machine and employing the following parameters: 180 kV, 15 mA, 0.5 mm Cu + 0.5 mm Al filtration. The two different rates were obtained by employing focal distances of 21 and 15 cm. The larvae were exposed to 40 kR in both cases.

In the second experiment larvae were exposed to X-rays using two different filters and the same technical parameters as in the first experiment. The range of exposure was 10 to 50 kR. One batch of larvae was irradiated employing 0.5 mm Cu + 0.5 mm Al filtration and an exposure of 280 R/min; the other batch was irradiated using 2.5 mm Al filtration and an exposure of 795 R/min, the focal distance being 21 cm in both instances.[1]

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[1] In fact, in this experiment both quality of radiation and dose-rate were changed. This design is justified by the results obtained in the first experiments, where it was shown that dose-rate was essentially without influence, vide infra.

TABLE I. EFFECT OF DOSE-RATE ON LARVAL DEVELOPMENT

	3 days after infection		6 days after infection			10 days after infection
	L_{III} exs.		L_{III-IV}	L_{IV}	Total	L
Exposure 250 R/min	211	Insignificant difference between 250 and 500 R $t = 0.54$ $P < 0.10$	48	69	117	0
	379		17	19	36	0
	136		39	23	62	0
	77		8	43	51	0
	114		17	21	38	0
	917				304	
	L_{III} exs.		L_{III-IV}	L_{IV}	Total	L
Exposure 500 R/min	60	Insignificant difference between 500 R and controls $t = 0.93$ $P < 0.10$	22	32	54	0
	124		7	17	24	0
	268		16	20	36	0
	18		3	19	22	0
	249		5	30	35	0
	719				171	

TABLE I. (contd.)

	3 days after infection	6 days after infection	10 days after infection
	L_{III} exs.		L_V
Control: unirradiated larvae	108	Insignificant difference between 250 and 500 R $t = 1.67$ $P < 0.10$	148
	257		0
	457		14
	158		43
	120		4
	1100		209

L_{III} exs. = third-stage exsheathed larvae.
L_{IV} = fourth-stage larvae.
L_{III-IV} = intermediary stage between the third and fourth stages.
L_V = fifth-stage larvae.

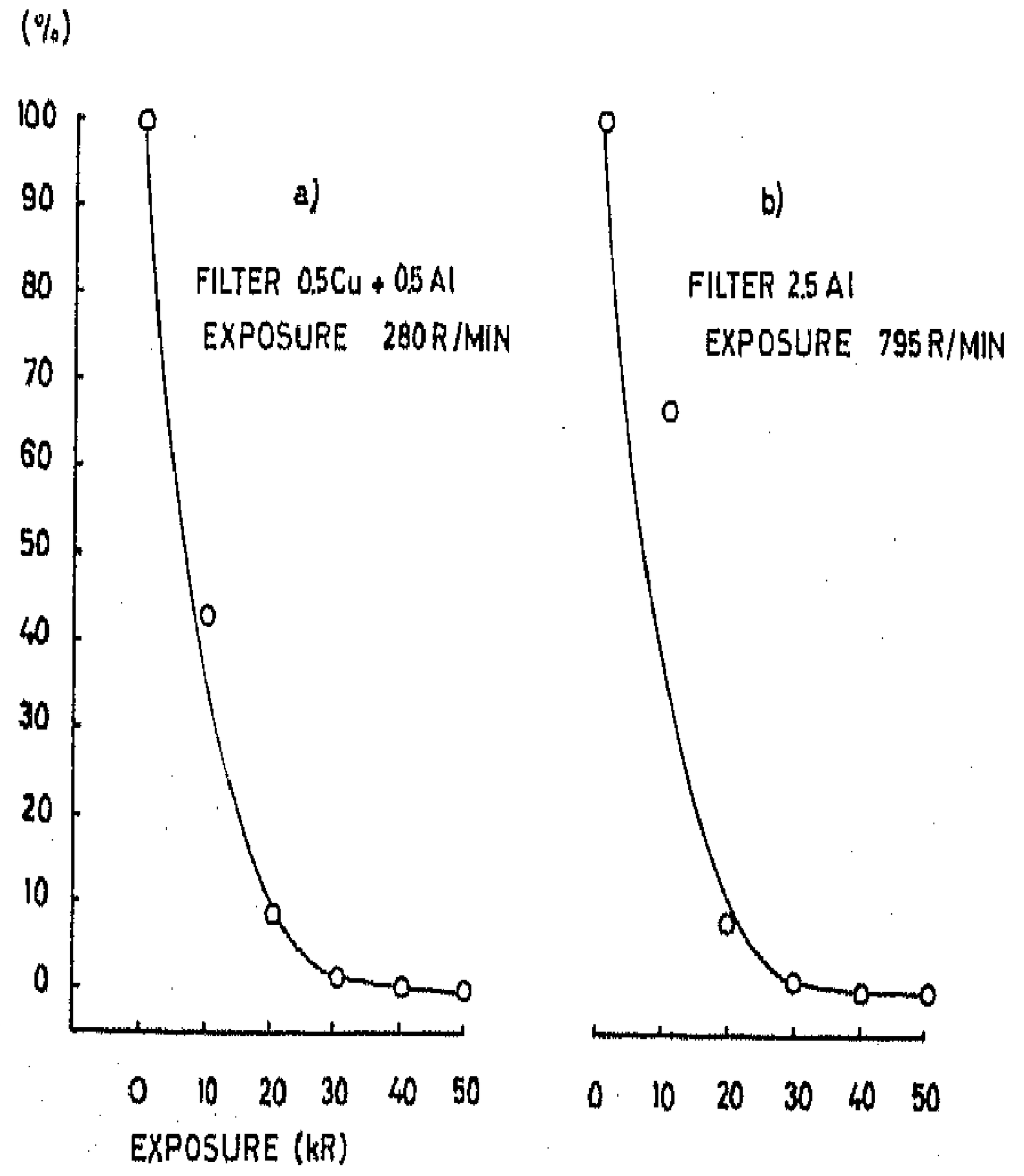

FIG. 6 a), b). The effect of different X-ray exposures and filtration upon infective larvae of Dictyocaulus viviparus.

The larvae were irradiated in aqueous suspension in a Plexiglas dish. On the second day after exposure the irradiated larvae were given orally to guinea pigs, weighing from 280 to 300 g, at a dose of 5000 larvae per animal. At the same time a control group of guinea pigs was infected with non-irradiated larvae which were recovered and kept under identical conditions to those of the irradiated larvae.

The guinea pigs were killed at 3, 6 and 10 days, respectively. The lungs were torn to pieces and macerated overnight in saline at a temperature of 37°C. On the following day the tissue suspension was strained through a sieve and centrifuged. The resultant sediment was spread on a glass slide in a thin layer and examined under the microscope at low magnification. In all instances the whole sediment was subjected to the treatment.

Results

1. No significant difference was found between the exposure to 250 R/min and to 500 R/min, the larvae being exposed to 40 kR in both experiments. This is shown by the counts of larvae recovered from the guinea-pig lungs. The larval counts and the stage of larval development are presented in Table I and Fig. 6a.

On the third day after infection there were no significant differences either between the two groups of irradiated larvae or between the irradiated and untreated larvae.

2. Likewise, the results obtained with the two exposures, due to different filtration, did not show any substantial differences in the degree of larval establishment and development. The groups irradiated with 10 kR showed a change in the male/female ratio on behalf of the females. From the irradiation dose of 20 kR onwards only female larvae developed.

Detailed results are presented in Tables II and III indicating total larval counts in individual groups, and in Fig. 6b.

TABLE II. EFFECT OF FILTRATION (Cu and Al) ON LARVAL DEVELOPMENT
(Filtration = 0.5 mm Cu + 0.5 mm Al — exposure: 280 R/min[a])

Radiation exposure (kR)	L_{III-IV}	L_{IV}	L_V	sex in L_V ♀	♂	♀/♂ ratio	Test of significance
Untreated	0	1	738	377	261	1.04 : 1	
10	1	1	319	256	63	4.06 : 1	Insignificant difference between untreated larvae and the 10 kR group t = 1.51; 0.2 <P < 0.1
20	1	2	65	65	0	♀ only	Highly significant difference between untreated larvae and the 20-kR group t = 2.57; 0.02 < P < 0.01
30	2	0	3	3	0		Highly significant difference between untreated larvae and the 30 kR group t = 3.04; 0.01 < P < 0.005
40	1	0	0	0	0		
50	3	0	1	1	0		

[a] There were ten guinea pigs in each group.

TABLE III. EFFECT OF FILTRATION (Al only) ON LARVAL DEVELOPMENT
(Filtration: 2.5 mm Al – exposure: 795 R/min[a])

Radiation exposure (kR)	L_{III-IV}	L_{IV}	L_V	Sex in L_V ♀	Sex in L_V ♂	♀/♂ ratio	Test of significance
Untreated	0	2	117	78	99	0.78 : 1	
10	0	0	120	83	37	2.24 : 1	Insignificant difference between untreated larvae and the 10-kR group $t = 0.99$; $P < 0.10$
20	0	1	13	13	0	♀ only	Highly significant difference between untreated larvae and the 20-kR group $t = 3.32$; $0.02 < P < 0.01$
30	0	1	1	1	0		
40	2	0	0	0	0		
50	0	0	0	0	0		

[a] Five guinea pigs were used in each group.

Discussion and conclusions

In agreement with other authors, Jennings et al. [4], Lucker and Vegors [6], Urquhart et al. [5], it was established in this study that different dose-rates do not vary in their effect on the development of Dictyocaulus viviparus in guinea pigs. The second part of the experiment presented evidence that no significant differences are produced in this sense, even if qualitatively different kinds of radiation, obtained by different filtration, are employed. With the focal distance and other technical parameters being unchanged, different filtration resulted
(a) in the radiation of two different qualities (effective wave-length for 2.5 mm Al was about 0.21 Å and for 0.5 mm Cu it was about 0.155 Å, disregarding 0.5 mm Al); and
(b) consequently, in two different exposures (795 R/min and 280 R/min).

The use of increasing X-ray doses, the comparison of the results and the construction of curves showed that the qualitatively different conditions of irradiation did not differ in their biological effects on infective larvae of Dictyocaulus viviparus. Therefore, it does not appear that the X-ray component which is normally filtered off by a commonly used filter made itself felt to a larger extent in the case of Dictyocaulus viviparus larvae, although soft radiation is generally considered to be biologically more effective.

From the results obtained in this study, as well as from the data reported in literature, it appears that the most important factor in X-irradiation of parasites is the total dose of radiation. Apparently, in phylogenetically low-standing parasitic worms neither the dose-rate per minute nor the X-ray quality (at least over an effective wave-length range of 0.155 to 0.21 Å) will be of any decisive value. This finding is of practical importance for the further utilization of X-ray machines for the preparation of radiation vaccines.

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IMMUNE RESPONSE OF YOUNG ANIMALS TO INFECTION WITH X-IRRADIATED AND NORMAL NEMATODE LARVAE

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Abstract

IMMUNE RESPONSE OF YOUNG ANIMALS TO INFECTION WITH X-IRRADIATED AND NORMAL NEMATODE LARVAE. Two problems associated with the vaccination of young animals are: (a) the difficulty of producing a degree of useful immunity before the host is subjected to a pathogenic level of infection; (b) the possibility that young animals may be incapable of developing a good immune response or that exposure to infection or vaccination in early life may affect the subsequent immune response in adult life.

These two topics are reviewed in the light of field and experimental evidence in farm animals. Finally, some recent experimental results on immunological unresponsiveness in the young rat to *Nippostrongylus brasiliensis* infection are presented and its implications discussed.

The fact that young animals are often exposed to significant infections of helminths while they are still very young presents two major problems in any scheme of artificial immunization.

The first is obvious and foreseeable and is simply the difficulty of producing a degree of useful immunity before the animals are subjected to pathogenic levels of infection. The second is less obvious but potentially very significant, namely, the possibility that young animals may be incapable of developing a good immune response because of some type of immunological immaturity.

These problems do not arise to any great degree with the commercially available irradiated vaccine against the bovine lungworm *Dictyocaulus viviparus*; in most parts of Europe dairy calves, the primary consumers of the vaccine, are born indoors and do not go to grass until April or May when they are four to six months old; by this time, vaccination is completed and immunity has been established. Over the past few years, however, large numbers of suckling beef calves at grass have also been vaccinated, with apparent success. In a recently completed study of on-pasture vaccination Downey (personal communication, 1967) has concluded that it would be effective under most circumstances; he stated that it would be undesirable to vaccinate in the presence of heavy infestation and that the best time would be March, April and early May.

The vaccination of lambs against *Dictyocaulus filaria* has been studied by Jovanovic and co-workers in Yugoslavia [1]. For practical considerations it is necessary in many instances that the first dose of vaccine to lambs be given at six weeks of age. They therefore double-vaccinated groups of lambs beginning at 18, 16, 8 and 6 weeks of age with 1000 and 2000 irradiated larvae and concluded, on the basis of faecal larval counts subsequent to challenge infection, that the degree of resistance was 95,

TABLE I. NUMBERS OF ADULT H. contortus RECOVERED AT AUTOPSY OF VACCINATED AND CONTROL MERINO LAMBS
(Each sheep was challenged with 10 000 larvae)

Merinos aged 6½ months (Reared on infected pasture)		Merinos aged 6½ months (Reared indoors)		Merinos aged 24 months (Reared indoors)	
Vaccinates	Controls	Vaccinates	Controls	Vaccinates	Controls
3375	7400	3900	3000	7	7
4975	2025	5575	5250		
1500	700	775	2975		
4425	3375	1650	3775		
8800	2475	1400	4000		
400	5600	750	3650		
		1475	100		
Mean: 3913	Mean: 3596	Mean: 2218 (N. S.)	Mean: 3250	Mean: 61 ± 69	Mean: 2250 ± 1629

91, 63 and 22%, respectively. Unfortunately, the significance of these results is obscured by the fact that the two younger age groups received a much heavier challenge than the two older groups. In a subsequent paper [2], in which the value of vaccination in an endemic area was studied, grazing lambs of two to four months of age were immunized. A proportion of these lambs was already naturally infected before vaccination commenced. Despite this, it was concluded at the end of the grazing season that the vaccine had conferred a significant degree of immunity both in reducing the percentage of infected animals and the size of the worm infections in the two groups. Nevertheless, the degree of protection was apparently not as high as that obtained after vaccination of worm-free lambs of comparable age.

The results of attempts to develop a vaccine against Haemonchus contortus infection in lambs were summarized in a previous report of this series [3] and have since been published in extenso [4-6]. In essence it was possible to protect Blackface sheep aged over six months against a challenge of 10 000 to 50 000 H. contortus larvae using two doses of X-irradiated larvae; the same regime in two-to-three-months-old lambs was completely unsuccessful.

Since then it has been possible to make some observations on the immune response of East African Merino lambs, both to natural infection and to vaccination [7]. The results of these may be summarized as follows:

1. Attempts to vaccinate pregnant ewes or adult male sheep reared from birth in an endemic area were completely unsuccessful. In these experiments sheep, while still at grass, were treated with thiabendazole[1] before each vaccination.
2. This apparently poor immunological response of adult Merino sheep reared in an endemic environment was confirmed in a second experiment. Two groups of 12 adult male sheep, grazing naturally infected pasture, were treated with thiabendazole 7 and 30 days, respectively, before each sheep was challenged with 10 000 larvae. From the time of treatment onwards they were brought indoors. A third group of 24 adult males were brought indoors on the day of challenge; 12 were autopsied and had a mean worm burden of 850 ± 480; each of the remainder was also challenged with 10 000 larvae. At autopsy, 30 days later, the mean worm burden of sheep in the three groups was 1979 ± 633, 1664 ± 583 and 1958 ± 1570.

In the absence of a control group of adult sheep, which had been reared from birth in a worm-free environment, it is difficult to assess the precise degree of immunity which had developed in these three groups. Nevertheless, by comparison with worm-free adult Blackface sheep [5] which develop a similar number of worms after a single experimental infection of 10 000 larvae, it appeared that in this particular instance, at least, Merino sheep had not acquired any significant immunity to reinfection despite repeated exposure to infection since birth.

Three groups of Merino sheep were double-vaccinated with X-irradiated larvae and challenged one month later with 10 000 larvae. The ages and histories of the three groups are shown in Table I. It is apparent that only the worm-free sheep aged two years were protected and that this protection was highly significant.

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[1] Thibenzole - Merck Sharp and Dohme Ltd.

From these experiments one might tentatively conclude that:

(a) East African Merinos only become immunologically responsive to Haemonchus infection at some point between 7 and 24 months of age.

(b) This immunity may be temporary and largely independent of any existing adult infection or alternatively fails to develop under field conditions because of exposure to infection during the long immunologically unresponsive period.

In a previous report [8] Miller discussed the possibility of developing X-irradiated vaccines against hookworm disease and reviewed current progress in the development of a vaccine against Ancylostoma caninum infection in the dog. Since then considerable progress has been made in the development of this vaccine and information is now available on its use in young pups [8]. It is well known that pups born in an endemic area are invariably prenatally infected with A. caninum larvae and may also acquire post-natal infection in the colostrum of their dam [9]. These larvae mature within 3 weeks of birth and frequently cause morbidity and mortality. In this situation one might reasonably anticipate a degree of immunological unresponsiveness in pups which might subsequently persist in adult life. Despite this, Miller has shown that if pups are treated with a suitable anthelmintic soon after birth they may be immunized, starting at four weeks of age. The degree of protection produced, i.e. of the order of 85% reduction in worm burden, is similar to that obtained in pups of the same age born to uninfected dams.

Miller has also shown that pups not prenatally infected may be immunized to a significant degree, even when vaccination is commenced as soon as three days after birth, although the degree of protection (51%) is not so high as when vaccination is started at four weeks (85%), three months (88%), or when adult (90%).

From the limited information summarized above, and from general considerations, one might conclude that very young animals cannot be as successfully immunized as older animals. In particular, the sheep appears to have a prolonged unresponsive period [4,5,10] to H. contortus infection, the length of time depending on the breed [7]. In this host-parasite relationship, it is also possible that early infection, naturally acquired, may permanently impair the immune response in later life.

The possibility of immune unresponsiveness is of considerable practical importance in both human and veterinary parasitology [11-13], since both hosts are commonly exposed to helminth infection in the first few weeks of life. To provide a background of experimental fact before investigating this phenomenon in the field, the authors are currently engaged in studying the immune response of the young rat to Nippostrongylus brasiliensis infection [14] and the following summary reviews the results to date:

In adult rats infected subcutaneously with 500 or more N. brasiliensis larvae, four phases of infection may be distinguished. These are:

(a) Loss Phase 1 (LP1)

This occurs in the first day of infection and is caused by the death of a proportion of the injected larvae before they reach the lungs.

(b) Plateau Phase (P1)

Following the establishment and maturation of the parasites in the intestine by the fourth day, the population remains static until about the 11th day.

(c) Loss Phase 2 (LP2)

From the 11th day onwards there is a rapid exponential loss of worms from the intestine at a rate of about one log unit in five days.

(d) Threshold Phase

The exponential loss terminates around day 18; a small residual worm burden remains in the intestine and may persist for long periods.

If rats are subsequently reinfected they are immune, in that the proportion of larvae lost during LP1 is increased, the plateau terminates by days 4 to 6, and the reaction rate of LP2 is increased. Again a "threshold" number of worms persists.

If young rats of one to six weeks of age are infected with 500 larvae a different sequence of events ensues, in that LP2 does not occur, the 'plateau phase' persisting for at least seven to eight weeks. Since the rats have been adult for several weeks by this point it is apparent that the unresponsiveness is not due to immunological immaturity alone.

When doses greater than 500 larvae are used a belated and partial self-cure occurs around day 17 but terminates prematurely when a threshold of 200 worms remains in the intestine.

The author considers that these two observations may be explained by assuming that baby rats are inefficient at antigen processing or antibody production. When the quantity of antigen is insufficient, either because of a small primary infection or as a result of worm expulsion, the production of critical antibody is not maintained or is insufficient to initiate a necessary cellular response.

This hypothesis is insufficient to account for the persistence of the plateau phase of 200 to 250 worms during the adult life of the rat. One may eliminate the induction of classical tolerance as an explanation, since it has been shown that adult unresponsive rats harbouring an infection of 200 worms developed an immune response when they were reinfected. This was primary or secondary in type, depending on the size of the original infection. Rats in which no self-cure occurred after the first infection developed a primary response; rats in which partial self-cure occurred after the first infection show a secondary response.

The author has considered three possible explanations for this type of adult unresponsiveness. These are:

1. It has been shown that a primary immune response of young animals may be restricted, e.g. confined to the production of gamma M [15] or, as has been shown by Binaghi et al. [16], deficient in precipitin but normal in anaphylactic antibody production. In the author's system it has been demonstrated that young rats infected with 500 to 800 larvae produce reaginic antibody to N. brasiliensis antigen, although showing no self-cure and having no demonstrable protective antibody. At 3 to 4 weeks after infection the serum titre of reaginic antibody in young rats

is equal to that of similarly infected adult rats and the young rats are equally susceptible to shock by intravenous *N. brasiliensis* antigen injection [17].

It therefore seems possible that in the young rat infected with *N. brasiliensis* the immune response is limited to a particular class of immuno-globulin and that the critical antibody is not produced. If this occurs it may subsequently interfere with the full expression of the normal immune response in adult life.

2. Antigen production by the adult worms may cease or diminish greatly while the rat is still immature so that there is no stimulus of critical antibody once this capacity matures.

3. The existence of an adult worm infection during the immunologically immature period of the rats' development may produce a state of exhaustion of a particular cellular response which is a necessary component of the self-cure reaction [18].

These observations are still in their early stages but demonstrate in at least one helminth infection that the immune reaction of the young host is deficient and that its subsequent response to reinfection may be altered.

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RELATIVE ROLE OF EGGS AND ADULT WORMS IN DEVELOPMENT OF LIVER DAMAGE IN MICE INFECTED WITH SCHISTOSOMA MANSONI

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Abstract

RELATIVE ROLE OF EGGS AND ADULT WORMS IN DEVELOPMENT OF LIVER DAMAGE IN MICE INFECTED WITH SCHISTOSOMA MANSONI. During the acute phase of S. mansoni infections, significant deviations in liver function tests were demonstrated and were related to egg deposition. Since exposure of cercariae to gamma irradiation have shown that a dose of 3000 rep prevents the developing worms from producing eggs, additional experiments were designed to determine whether relatively heavy worm loads without egg production would result in significant deviations in liver functions either before or after treatment.

Mice were exposed to 10 000 cercariae which had been irradiated at 3000 rep and were divided into two groups. The animals of the first group were treated during the ninth week after exposure to infection and those of the second group were left untreated. At necropsy the worms recovered from both groups were stunted and there was no evidence of schistosome eggs in any of the organs of the mice. Infection with irradiated larvae produced only a transitory increase in transaminase levels (SGP-T and SGO-T) during the third week after exposure. No increases or significant group differences were observed in the transaminase values after the worms had reached adulthood. However, there was a significant increase in gamma globulin as infection progressed. These results indicate that the egg is the main pathogenic agent in schistosomiasis and add considerable evidence to the view that treatment can prevent the establishment of hepatic fibrosis.

INTRODUCTION

The relative role of eggs and adult worms in the production of hepatosplenic schistosomiasis is still a subject of great controversy. Until this question is resolved, many clinicians seriously doubt the advisability of treating patients with specific drugs which might aggravate the symptoms as a result of the death of the parasites and their decomposition within the intrahepatic portal branches [1, 2]. Numerous publications emphasize the importance of the role of dead worms as the real origin of Symmers liver fibrosis resulting from obstruction of the intrahepatic portal radicles followed by focal necrosis due to tissue ischaemia. Jaffe [3] described a process of infiltrative reaction found around dead schistosomes in liver tissue and stated that live parasites may be responsible for local endovasculitis and diffused lesions through the liberation of toxins. Day [4] related extensive hepatic lesions in tissue preparations to the presence of dead worms causing thrombosis in the portal veins. Several investigators [5 - 8] have also suggested that liver damage is produced by dead schistosomes after specific therapy. Souidan [9] concluded that the liberation of metabolites, probably histamine, from the dying and dead worms causes a further rise in the portal pressure which leads to additional enlargement of the liver and spleen and another rise of portal pressure. However, an equally impressive series of observations [10, 11] indicates that the lesions produced by dead schistosomes are focal and of minor importance in the genesis of human liver fibrosis. Salem [12] reported that specific treatment was beneficial to the patients and observed no damaging effects to the livers of 90 patients with hepatic fibrosis during or following therapy.

Studies conducted with experimental animals have also provided inconclusive results. Fairley and his co-workers [13 - 15] demonstrated a "verminous phlebitis" in the liver and pancreas of goats hyperinfected with *Schistosoma spindale* which led to death, and they concluded that dead parasites are actively pathogenic. Meleney et al.[16] reported that dead worms stimulate thrombosis and an intense perivascular reaction which leads to scar formation, recanalization of the vessels, and disappearance of worms. Menezes [17, 18], using enormous amounts of lyophilized adult *S. mansoni* worms, produced lesions in the livers of rats and rabbits similar to those of Symmers fibrosis, and he stated that more emphasis should be placed on the role of dead worms in the production of lesions. Coelho [19] found that mice developed severe liver damage after receiving specific therapy. Conversely, DeWitt and Warren [20] described a syndrome in mice resembling hepatosplenic schistosomiasis in man and concluded that this syndrome is due mainly to the presence of large numbers of eggs which incite granulomatous reactions and obstruct the portal blood flow. Warren [21] found that adult worms alone do not produce this syndrome. He also observed that even light infections of only one or two pairs of worms resulted in hepatosplenic schistosomiasis. These results were confirmed and extended by Sadun and Williams [22], who observed marked liver function changes in mice infected with only a few schistosomes.

The World Health Organization's Scientific Group on Research in Bilharziasis (Chemotherapy), in a report to the Director General in 1964, stated that studies using different modern techniques for the detection of liver damage are urgently needed to increase our knowledge of the pathogenesis of schistosomiasis in relation to chemotherapy, and recommended that all parameters available for the study of the development of liver damage, including liver function tests, should be used.

The use of ultramicrobiochemical techniques has made it possible to obtain an accurate quantitative estimate of serum components in mice before and at various intervals after infection with parasites [23, 24]. During the acute phase of *S. mansoni* infections significant deviations in liver function tests were demonstrated. These abnormalities appeared to accompany egg deposition and occurred even in mice harbouring just a few worms. The onset of hepatosplenic schistosomiasis appeared to be related to the intensity and duration of infection [22].

Experiments to determine the effect of exposure to gamma radiation on the ability of cercariae to reach maturity in mice have shown that with doses up to 2000 rep a few worms were able to mature and to produce eggs. However, no eggs could be recovered in the stools or in the intestinal tissues of mice exposed to cercariae which had been irradiated at 3000 or more rep [25].

A non-antimonial compound was recently reported to have effective schistosomicidal activity against adult *S. mansoni* worms in vitro in a concentration of 10 μg/ml. In a concentration of 1 μg/ml female schistosomes did not lay eggs. Subsequent studies in mice infected with *S. mansoni* revealed no living worms at necropsy when the drug was administered orally at a dose of 100 mg/kg for 10 consecutive days [26, 27]. The full therapeutic course of this compound appeared to have no significant hepatotoxic action in normal or infected mice [28].

In view of these considerations, studies were begun to (a) determine whether liver damage resulting from infection with *S. mansoni* in mice is of a transitory character, (b) assess the relative role of eggs and adult worms in the production of hepatosplenic schistosomiasis, and (c) evaluate the effect of treatment before and after significant deviations in some liver function tests.

MATERIALS AND METHODS

Young male albino mice (Inbred Charles River strain) weighing 18-23 g at the beginning of each experiment were used. The experimental mice were exposed to single doses of 100 or 200 non-irradiated cercariae or to 10 000 irradiated cercariae of the Puerto Rican strain of *S. mansoni*. The techniques used for the maintenance of the life cycle and the collection of cercariae have been described previously [25]. At necropsy the Perf-O-Suction technique [29, 30] was used for recovering the schistosomes.

Liver function tests were limited to those which in previous studies indicated consistent and significant abnormalities resulting from schistosomiasis [22]. The levels of serum glutamic pyruvic transaminase (SGP-T), serum glutamic oxaloacetic transaminase (SGO-T), and total protein were determined. In addition, serum electrophoresis was conducted to estimate the relative amounts of serum protein fractions. These tests were performed on all animals before infection and at weekly intervals thereafter throughout each experiment. A detailed description of the techniques used, the values to be expected in uninfected mice and some of the variables which might alter these values have been reported elsewhere [31].

A "Gammacell 220" cobalt-60 irradiator described by Rice and Smythe [32] was used as the source of gamma radiation. This 1300 Ci source delivered a dose-rate of approximately 1025 rep/min as calculated by the Fricke ferrous sulphate method of Weiss et al. [33]. Pyrex glass tubes (16 mm × 90 mm) containing *S. mansoni* cercariae were placed in a test-tube holder to rotate on its own axis while the test tubes were rotated on their axes. This method insured a uniform exposure to radiation of the cercariae in each of the test tubes. The cercariae were exposed to approximately 3000 rep[1] [25].

Treatment with a nitrothiazole derivative, 1-(5-nitro-2-thiazolyl)-2-imidazolidione[2] was carried out as follows: the drug was suspended in

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[1] In the irradiation of aqueous suspensions of parasites the roentgen and rep are almost equivalent. For recommendations on units, see National Bureau of Standards Handbook (1963) 3.3.

[2] Ambilhar, Ciba, S.A., Basel, Switzerland.

TABLE I. SERUM TRANSAMINASES VALUES (units/ml) BEFORE AND AFTER TREATMENT IN MICE EXPOSED TO 100 Schistosoma mansoni cercariae

Test	Group	Mice (No.)	Mean values at given week after exposure														
			0	1	2	3	4	5	6	7	8[a]	9	10	11	12	13	14
SGO-T	Treated	33	55	51	60	71	87	62	91	105	105[a]	100	80	64	62	65	43
	Untreated	18	51	47	63	61	64	65	104	88	102	-	-	-	-	-	-
SGP-T	Treated	33	23	20	29	35	44	33	60	75	38[a]	60	47	33	28	25	35
	Untreated	18	23	20	29	32	30	28	89	75	84	-	-	-	-	-	-
SGP-T/SGO-T	Treated	33	0.43	0.39	0.47	0.49	0.50	0.50	0.63	0.70	0.33[a]	0.64	0.51	0.50	0.46	0.40	0.32
	Untreated	18	0.43	0.44	0.48	0.52	0.48	0.44	0.73	0.82	0.80	-	-	-	-	-	-

[a] Beginning of treatment.

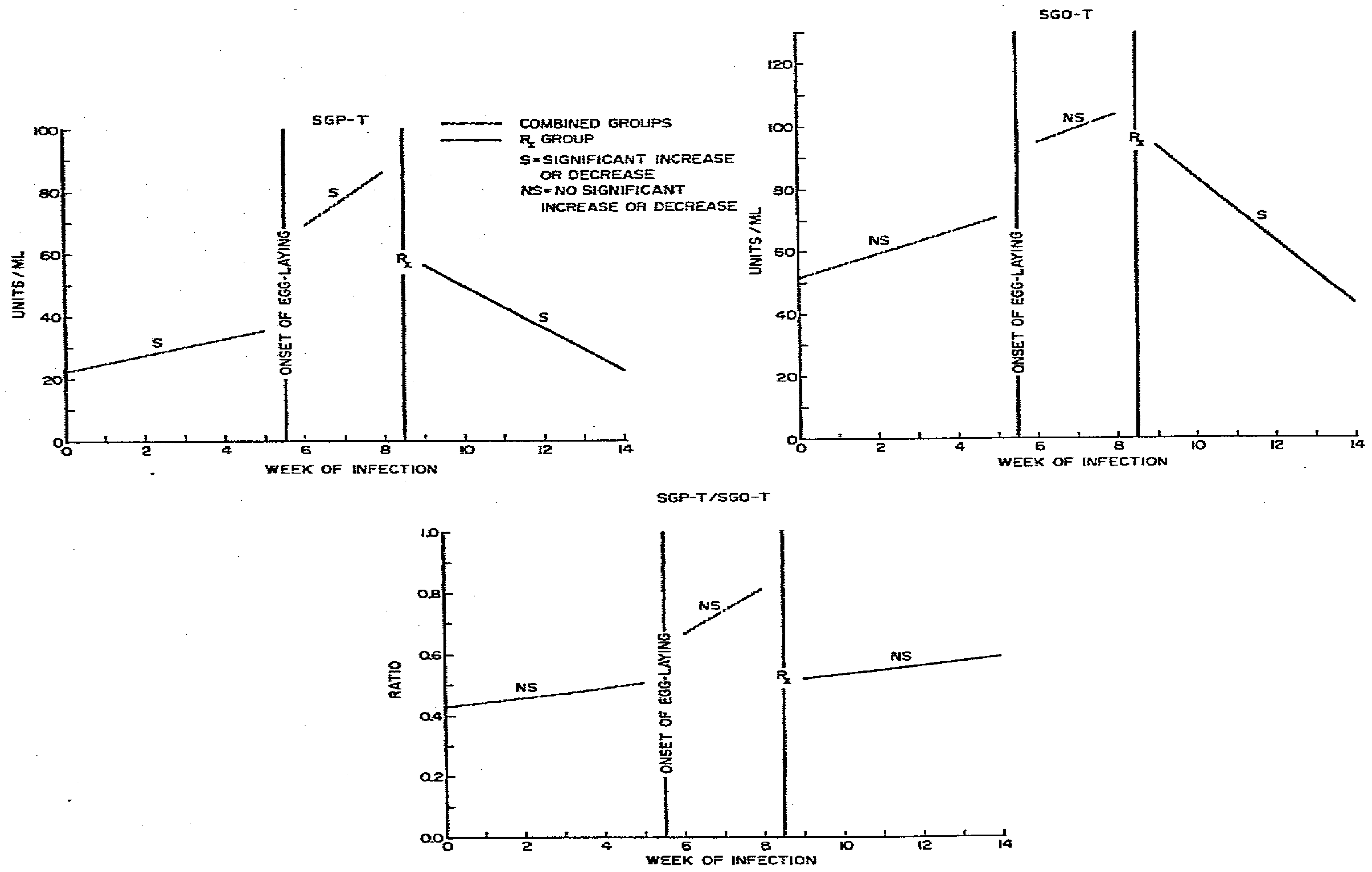

FIG. 1. Least squares regression lines calculated from weekly mean levels of serum transaminases of untreated and treated mice exposed to 100 S. mansoni cercariae.

TABLE II. SERUM PROTEIN VALUES (g%) BEFORE AND AFTER TREATMENT IN MICE EXPOSED TO 100 Schistosoma Mansoni cercariae

Test	Group	Mice (No.)	Mean values at given week after exposure														
			0	1	2	3	4	5	6	7	8[a]	9	10	11	12	13	14
Total Protein	Treated	33	5.4	5.6	6.0	6.0	6.0	5.9	5.8	5.8	5.9[a]	6.2	6.6	6.6	7.5	7.8	7.7
	Untreated	18	5.3	5.8	6.0	5.8	5.8	5.8	5.9	5.2	6.1	-	-	-	-	-	-
AG	Treated	33	1.24	1.03	0.89	0.95	0.91	0.90	0.98	0.97	0.71[a]	0.68	0.63	0.65	0.51	0.52	0.49
	Untreated	18	1.44	1.07	1.00	1.04	0.94	0.86	0.92	0.79	0.64	-	-	-	-	-	-
Albumin	Treated	33	2.98	2.83	2.78	2.90	2.84	2.76	2.84	2.87	2.44[a]	2.43	2.46	2.42	2.52	2.60	2.50
	Untreated	18	3.13	2.98	2.98	2.90	2.79	2.66	2.71	2.28	2.33	-	-	-	-	-	-
Alpha 1	Treated	33	0.52	0.63	0.72	0.72	0.70	0.69	0.61	0.80	0.63[a]	0.51	0.49	0.51	0.52	0.40	0.44
	Untreated	18	0.44	0.54	0.55	0.61	0.67	0.65	0.61	0.82	0.60	-	-	-	-	-	-
Alpha 2	Treated	33	0.54	0.62	0.67	0.70	0.66	0.77	0.65	0.56	0.65[a]	0.55	0.57	0.57	0.66	0.73	0.72
	Untreated	18	0.57	0.71	0.69	0.70	0.51	0.77	0.67	0.57	0.63	-	-	-	-	-	-
Beta	Treated	33	1.14	1.24	1.32	1.27	1.38	1.26	1.25	1.08	1.72[a]	1.81	2.02	1.84	2.13	2.45	2.40
	Untreated	18	1.05	1.33	1.40	1.23	1.40	1.26	1.44	1.14	1.82	-	-	-	-	-	-
Gamma	Treated	33	0.19	0.25	0.43	0.39	0.45	0.39	0.47	0.52	0.53[a]	0.89	1.06	1.20	1.71	1.60	1.64
	Untreated	18	0.12	0.25	0.40	0.34	0.45	0.45	0.48	0.37	0.68	-	-	-	-	-	-

[a] Beginning of treatment.

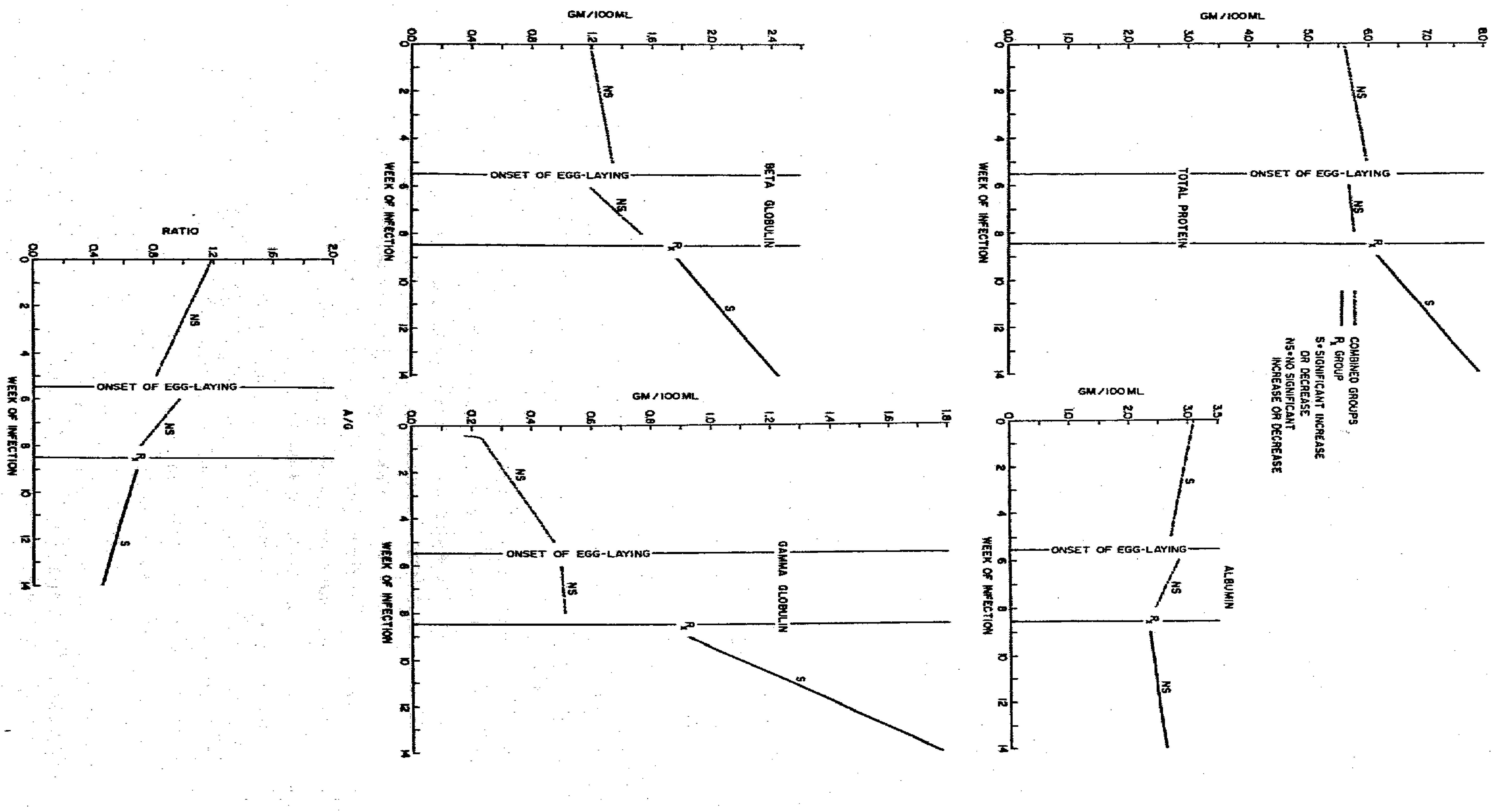

FIG. 2. Least squares regression lines calculated from weekly mean levels of serum proteins of untreated and treated mice exposed to 100 S. mansoni cercariae.

a 10% solution of methyl cellulose. Mice were treated by mouth with 100 mg of drug per kilogram of body weight each day for five consecutive days. The interval after infection at which treatment was begun varied with different experiments.

The method of least squares was employed for the study of trends (overall increases or decreases) in the weekly mean serum biochemical levels of the treated mice and of the untreated mice, or of the two groups combined. The period of study was broken down into appropriate intervals according to time of treatment and time of onset of egg-laying. The slope of the regression line for each interval was tested against a null hypothesis of zero slope by use of the t-test at the 0.05 level of significance. When each curve was broken down into the two or more periods for study, the resulting curve portions approximated linearity sufficiently to justify the use of a linear regression model.

For individual weeks, the serum biochemical levels of the treated mice were compared to those of the untreated mice by use of the Wilcoxon Two-Sample Rank Test [34, 35]. Reference to significant differences at any single week is based on the results of a Wilcoxon test, and any reference to a significant trend over a period of several weeks is based on the results of a regression-line slope test.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

RESULTS

A first experiment was performed to determine whether the damage produced as a result of egg deposition could be arrested or reversed by treatment. The mice were exposed to 100 cercariae each and divided into two groups. Treatment of the animals of the first group was initiated during the ninth week after exposure to infection. At necropsy, an average of 24.5 worms (14.7 males and 9.8 females) was recovered from the untreated animals, as compared to an average of 2.2 stunted worms (1.4 males and 0.8 females) in the treated group. There was no gross evidence of schistosome eggs in the tissues of any of the treated mice. There were slight but consistent overall increases in transaminase levels during the first five weeks of infection (Fig. 1, Table I) followed by a marked increase in transaminase levels after the fifth week (onset of egg-laying). The SGO-T and SGP-T levels continued to rise through week 8. After the eighth week, the transaminase levels of the treated animals showed a significant decrease and began a return to preinfection levels. The SGP-T/SGO-T ratios again revealed that the SGP-T changes were more marked than those of the SGO-T. In contrast to the serum transaminases, which returned to preinfection levels, the beta globulin, gamma globulin and total protein levels of the mice in the treated group continued to rise throughout the last six weeks of infection (Fig. 2, Table II).

A second experiment was designed to determine whether relatively heavy worm loads without egg production would result in significant deviations in liver functions either before or after treatment. Mice were exposed to 10 000 cercariae which had been irradiated at 3000 rep, and were divided into two groups. The animals of the first group were treated during the ninth week after exposure to infection, and those of the second

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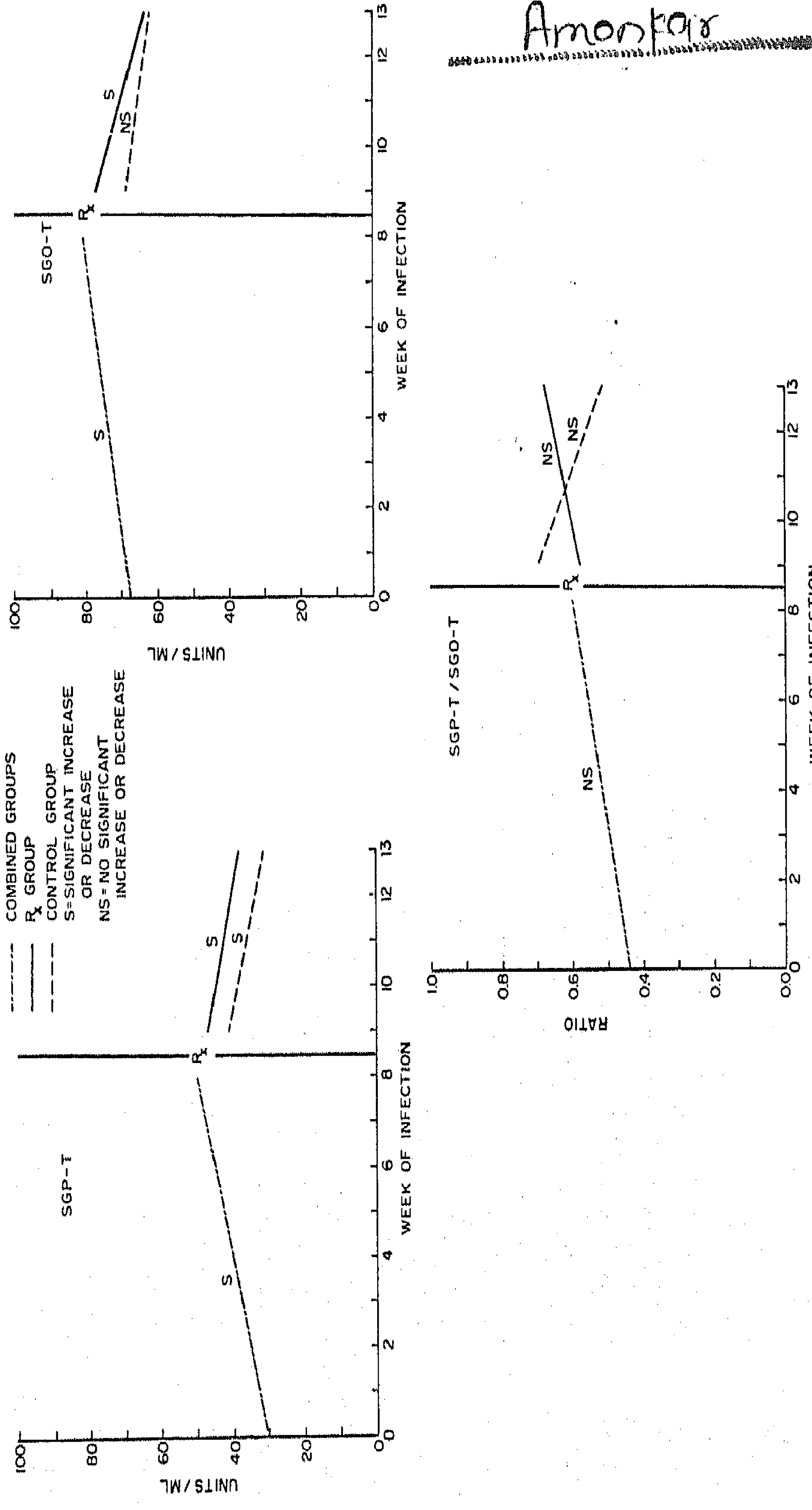

FIG. 3. Least squares regression lines calculated from weekly mean levels of serum transaminases of untreated and treated mice exposed to 10 000 irradiated *S. mansoni* cercariae.

TABLE III. SERUM TRANSAMINASES VALUES (units/ml) BEFORE AND AFTER TREATMENT IN MICE EXPOSED TO 10 000 *Schistosoma mansoni* cercariae IRRADIATED AT 3000 rep

Test	Group	Mice (No.)	Mean values at given week after exposure.													
			0	1	2	3	4	5	6	7	8[a]	9	10	11	12	13
SGO-T	Treated	25	73	66	50	67	71	68	65	84	85[a]	89	75	60	60	70
	Untreated	20	53	67	79	98	91	98	88	74	59	62	71	69	75	52
SGP-T	Treated	25	26	19	18	37	53	44	40	49	62[a]	41	56	38	40	39
	Untreated	20	23	32	35	80	51	52	34	36	28	39	35	43	38	25
SGP-T/SGO-T	Treated	25	0.37	0.29	0.37	0.56	0.55	0.65	0.63	0.59	0.72[a]	0.46	0.77	0.66	0.67	0.60
	Untreated	20	0.42	0.50	0.47	0.82	0.57	0.53	0.45	0.47	0.48	0.66	0.56	0.64	0.57	0.49

[a] Beginning of treatment.

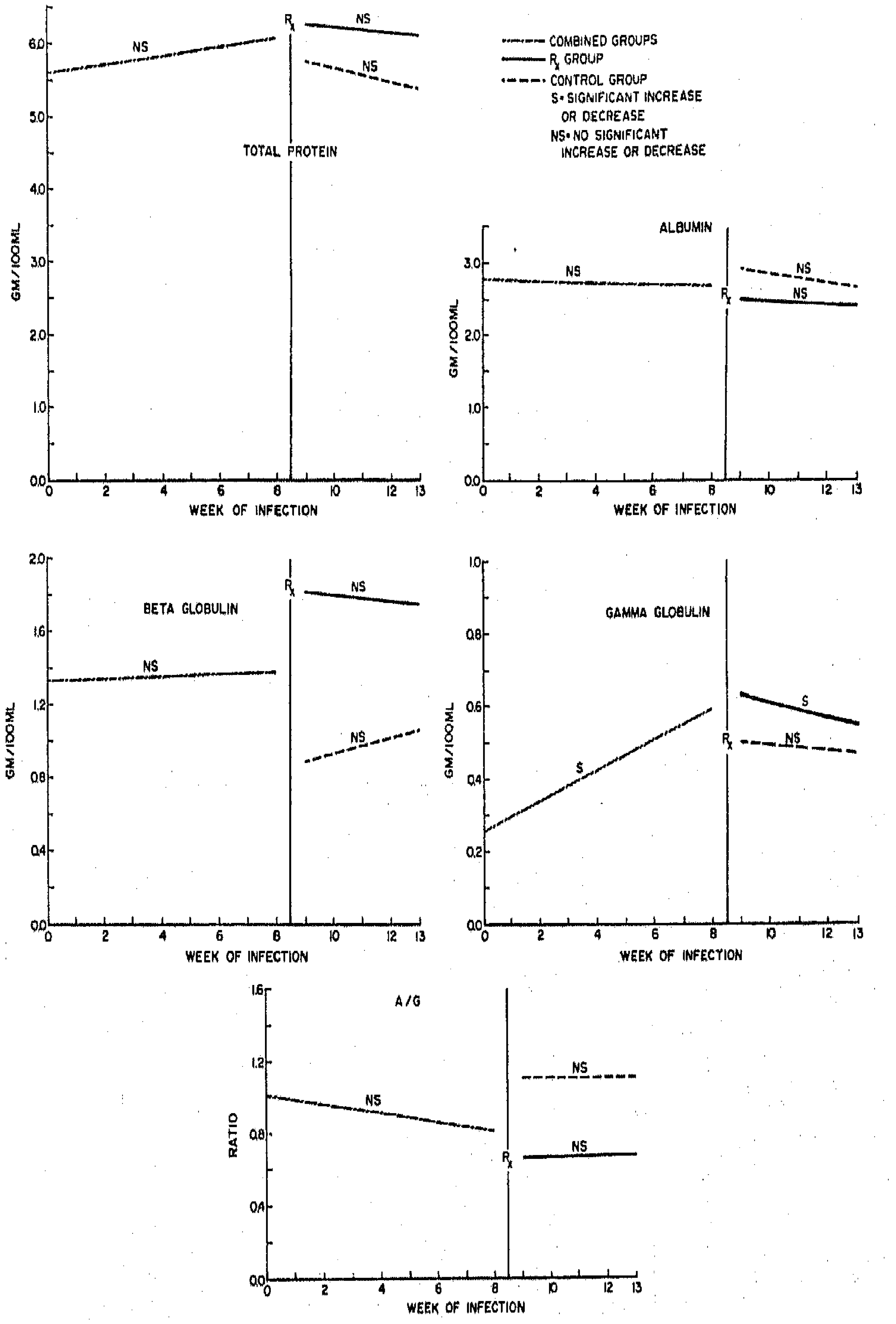

FIG.4. Least squares regression lines calculated from weekly mean levels of serum proteins of untreated and treated mice exposed to 10 000 irradiated *S. mansoni* cercariae.

group were not treated. At necropsy, an average of 29.5 worms (12.6 males and 16.9 females) was recovered from the untreated animals and an average of 13.1 worms (5.9 males and 7.2 females) from the treated group. The worms recovered from both groups were stunted and there was no evidence of schistosome eggs in any of the organs of the mice. As indicated in Table III and Fig. 3, no consistent significant group differences were observed in the transaminase values after the worms had reached adulthood. However, there was a transitory significant increase in transaminase levels of the combined groups during the second

TABLE IV. SERUM PROTEIN VALUES (g%) BEFORE AND AFTER TREATMENT IN MICE EXPOSED TO 10 000 Schistosoma mansoni cercariae IRRADIATED AT 3000 rep

Test	Group	Mice (No.)	Mean values at given week after exposure													
			0	1	2	3	4	5	6	7	8[a]	9	10	11	12	13
Total Protein	Treated	25	5.1	5.6	5.8	6.3	6.0	6.1	5.7	6.3	6.0[a]	6.3	6.3	6.0	6.4	6.1
	Untreated	20	5.7	5.7	5.9	6.1	5.6	5.7	5.9	5.7	5.8	5.4	5.7	5.5	5.0	5.6
A/G	Treated	25	1.02	0.82	0.87	0.74	0.80	0.73	0.68	0.73	0.62[a]	0.66	0.66	0.70	0.66	0.64
	Untreated	20	1.14	1.17	1.03	1.07	1.04	1.06	1.28	1.02	0.93	1.11	1.08	1.14	1.12	1.11
Albumin	Treated	25	2.58	2.56	2.78	2.71	2.61	2.60	2.37	2.76	2.31[a]	2.46	2.56	2.52	2.57	2.34
	Untreated	20	2.99	3.09	2.96	2.99	2.80	2.89	ND	2.85	ND	2.87	2.96	2.94	2.19	2.96
Alpha 1	Treated	25	0.39	0.52	0.53	0.64	0.56	0.69	0.70	0.58	0.63[a]	0.61	0.62	0.58	0.78	0.66
	Untreated	20	0.66	0.69	0.67	0.71	0.69	0.70	ND	0.70	ND	0.60	0.65	0.60	0.56	0.64
Alpha 2	Treated	25	0.55	0.63	0.65	0.81	0.72	0.74	0.69	0.84	0.78[a]	0.80	0.79	0.73	0.69	0.66
	Untreated	20	0.69	0.66	0.76	0.68	0.74	0.60	ND	0.66	ND	0.63	0.60	0.54	0.62	0.55
Beta	Treated	25	1.42	1.70	1.60	1.74	1.56	1.66	1.55	1.82	1.76[a]	1.82	1.82	1.60	1.88	1.87
	Untreated	20	1.05	0.96	1.05	1.13	0.94	1.01	ND	0.97	ND	0.87	0.95	1.03	0.95	1.08
Gamma	Treated	25	0.17	0.23	0.29	0.43	0.40	0.46	0.48	0.56	0.58[a]	0.63	0.59	0.62	0.58	0.54
	Untreated	20	0.32	0.37	0.41	0.42	0.45	0.45	ND	0.54	ND	0.49	0.54	0.44	0.38	0.50

[a] Beginning of treatment.
ND = Not done.

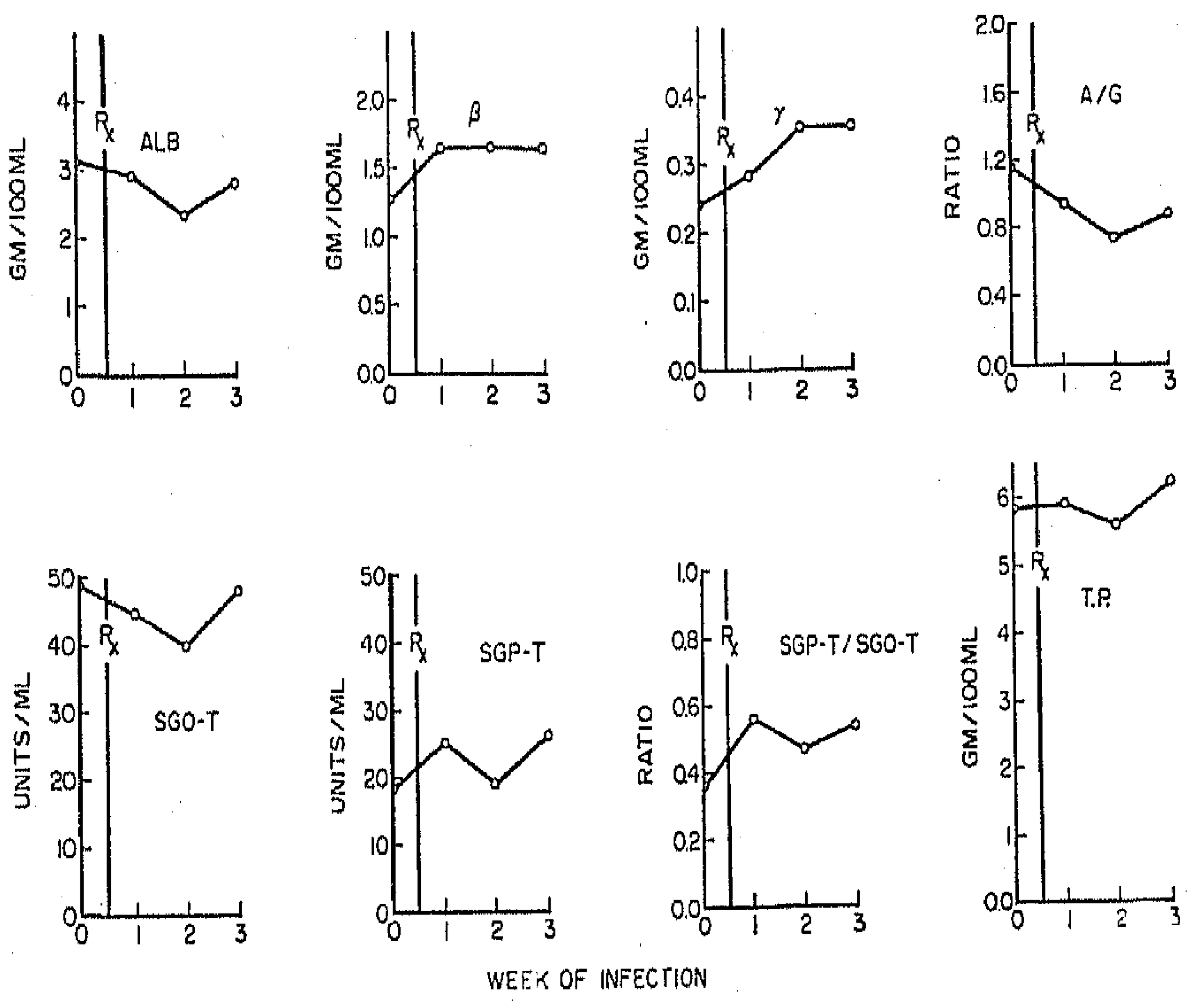

FIG.5. Serum biochemical levels of Ambilhar-treated, uninfected control mice.

TABLE V. MEAN SERUM TRANSAMINASES AND PROTEIN VALUES ON UNINFECTED MICE BEFORE AND AFTER TREATMENT

Test	Mice (No.)	Mean values at given week			
		0	1	2	3
SGO-T	26	49[a]	45	40	48
SGP-T	26	18[a]	25	19	26
SGP-T/SGO-T	26	0.35[a]	0.56	0.46	0.53
Total Protein	26	5.8[a]	5.9	5.6	6.2
A/G	26	1.16[a]	0.94	0.72	0.86
Albumin	26	3.15[a]	2.91	2.34	2.82
Alpha 1	26	0.76[a]	0.48	0.53	0.64
Alpha 2	26	0.46[a]	0.63	0.76	0.73
Beta	26	1.27[a]	1.64	1.65	1.63
Gamma	26	0.23[a]	0.28	0.35	0.35

[a] Beginning of treatment.

week after exposure (significant at weeks 3, 4 and 5), with a resulting overall increase in the SGO-T and SGP-T levels during the first eight weeks of infection. In both groups there was a significant increase in gamma globulin as the infection progressed up to the time of treatment (Table IV and Fig. 4). The gamma globulin levels of the treated mice were significantly greater than those of the untreated mice on three of the five post-treatment weeks (i.e. on weeks 9, 11 and 12).

A third experiment was designed to determine whether treatment itself in uninfected mice might have been responsible for some of the observed deviations in liver function tests. No significant changes (Table V and Fig. 5) were observed in any of the parameters studied up to three weeks following drug administration.

DISCUSSION

The extent of damage caused by adult schistosomes is largely unknown and much of the evidence is speculative. There is no proof as yet of inflammatory reactions set up in the venous system of man by the mere presence of living adult worms, although there is suggestive evidence to show that dead schistosomes in treated as well as untreated patients usually produce lesions of a focal character in the liver. It is possible that in cases in which liver damage is already extensive, and especially when the worm load is very high, specific therapy may result in complications through the shifting of dead or dying worms. However, in these situations the course of infection cannot be predicted if the patients are left untreated and oviposition is allowed to continue.

The results obtained with mice exposed to large numbers of irradiated cercariae support and extend these findings, suggesting that the lesions produced in the liver were not sufficient to bring about significant alterations in the liver function tests. Likewise, treatment and resultant reabsorption of dead worms did not bring about any detectable alteration of liver functions. It is of interest, however, that a significant increase in transaminase levels occurred in the mice exposed to irradiated cercariae during the early weeks of infection and was most evident at weeks 3 through 5. This agrees with histopathologic observations which indicated that a large percentage of irradiated schistosomula were stopped in the lungs where they elicited considerable reaction [36]. The elevation in transaminase levels might thus be related to the damage occurring in the lungs rather than in the liver [37].

The results of these and previous experiments leave little doubt that the egg is the main pathogenic agent in schistosomiasis. Eggs caught in the tissues elicit a marked cellular response which eventually results in the formation of fibrous tissue affecting the function of the organ. In experimental animals there is good correlation between the number of eggs in the tissues, the degree of host reactivity as expressed by size and cell composition of granulomas and the severity of organ damage. The present studies add considerable evidence to the view that chemotherapy can prevent the establishment of hepatic fibrosis. Since the primary role of the egg in the production of liver damage is well demonstrated, it seems logical that specific therapy by the suppression of egg-laying would prevent the development of marked tissue damage in cases treated early in the course of infection. This was true in the authors' experimentally infected mice. Furthermore, treatment also seemed to reduce the intensity of lesions of those treated later.

The World Health Organization's Scientific Group on Research in Bilharziasis (Chemotherapy), in a report to the Director General in 1964, concluded that there is some evidence that chemotherapy can prevent the establishment of hepatic fibrosis. The results of the author's studies with experimentally infected mice provide additional evidence in support of this statement.

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RESISTANCE PRODUCED IN RATS AND MICE BY EXPOSURE TO IRRADIATED PLASMODIUM BERGHEI*

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Abstract

RESISTANCE PRODUCED IN RATS AND MICE BY EXPOSURE TO IRRADIATED Plasmodium berghei. Rats infected with P. berghei parasitized erythrocytes exposed to doses up to and including 16000 R developed progressive parasitaemias with obvious parasite multiplication and reinvasion of RBC's. Some rats receiving parasitized RBC's irradiated at levels of 17000 and 18000 R did not develop progressive infections. Infections were aborted by exposure to irradiation at 19000 R or higher.

Inoculation with parasitized blood exposed to 20000 R stimulated a resistance to a challenge infection with non-irradiated parasites. The peak parasitaemias reached in the immunized animals were significantly lower than those of the non-immunized controls and reduction in parasitaemias began much earlier. The degree of acquired resistance induced by inoculation with irradiated parasites was influenced by the number of immunizing exposures before the challenging infection.

The acquired resistance induced by irradiated infected RBC's could be detected also in a more susceptible animal, such as the mouse. Twice-weekly inoculations of irradiated parasitized cells given 3, 5, and 10 times considerably extended the mean survival time after challenge; a significant number of mice survived otherwise lethal infections. In both rats and mice the degree of acquired immunity was directly proportional to the number of immunizing doses. Similar results were obtained when immunizations occurred at weekly intervals, or when given twice weekly. Likewise, no significant differences were detected when the challenge took place one or two weeks after the last immunizing dose.

INTRODUCTION

Attempts to produce immunity in rats and mice against Plasmodium berghei infections have been undertaken by several investigators with varying success. Although a sterile immunity was described in rats which had recovered from an infection with P. berghei Corradetti [1], attempts to produce artificial immunity with killed parasites have resulted in only partial protection. Following immunization of rats with a crude P. berghei antigen, Zuckerman et al. [2] obtained a moderate degree of resistance, whereas Corradetti et al. [3] did not. P. berghei-infected mice cured by drug therapy became resistant to a challenge infection, Box and Gingrich [4], Cox [5], Briggs et al. [6]. Temporary and partial protection was also achieved when "immune" rat serum was passively transferred to rats, Fabiani and Fulchiron [7] Bruce-Chwatt and Gibson [8], and to mice, Briggs et al. [9]. Similar results in rats and mice were obtained by the non-specific stimulation of the reticuloendothelial system, using bacterial endotoxins, Martin et al. [10].

The inconclusive results obtained with killed vaccines have been attributed to the loss or denaturation of a soluble antigenic fraction during the preparation of the vaccines. Furthermore, the parasites may have been too rapidly destroyed in the host to elicit a discernible protective effect. Sinton [11] postulated that the amount and rate of development of

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immunity to malaria may depend upon the amount and duration of antigenic stimulation. This suggestion was strengthened by the success reported recently by Weiss [12] in immunizing mice with a strain of P. berghei which had been rendered non-invasive by incubation in a tissue-culture medium containing hamster serum.

Irradiation of parasites interferes with their physiologic processes and frequently inhibits their normal development and multiplication. Although ionizing radiations inhibit avian malarial parasites in their normal multiplication, Bennison and Coatney [13], Rigdon and Rudisell [14], the reproductive processes are affected by a lower dose of radiation than the metabolic processes, Ceithaml and Evans [15]. Therefore, the use of irradiated parasites as vaccines might enable one to take advantage of the special immunological properties of living parasites while at the same time suppressing the pathogenic effects of the vaccine resulting from the reproductive capacity of the parasite involved.

A vaccine consisting of irradiated larvae was developed against the cattle lung worm Dictyocaulus viviparus, Jarret et al. [16 - 18] and is being produced commercially. Other successful vaccines using irradiated helminth parasites have been reported against Hæmonchus contortus, Urquhart et al. [19], Trichostrongylus colubriformis, Mulligan et al. [20], Tænia saginata, Urquhart [21], Uncinaria stenocephala, Dow et al. [22, 23], Ancylostoma caninum, Miller [24]. A marked degree of acquired immunity was induced also by the use of irradiated Trichinella spiralis larvae, Tyzzer and Honeij [25], Levin and Evans [26] and irradiated cercariae of Schistosoma mansoni, Villella et al. [27], Radke and Sadun [28] or S. japonicum, Hsü et al. [29]. Only infrequent attempts to immunize against protozoan infections with ionizing radiation have been reported, although Urquhart [30] obtained encouraging results with Eimeria tenella in chickens and Theileria parva in cattle.

Immunization by the use of irradiated malarial parasites has been reported by Ceithaml and Evans [15]. They found that chicken erythrocytes parasitized with Plasmodium gallinaceum exposed to 30 000 roentgen units (R) when injected into normal chickens conferred some degree of resistance to a challenge with normal parasites of the same strain. Recent studies, Corradetti et al. [31], indicated that essentially similar results could be achieved in rats by irradiation of the erythrocytic forms of Plasmodium berghei.

The present series of investigations was undertaken to study further the effect of ionizing radiation on the reproduction and survival of blood forms of the mammalian malaria parasite, Plasmodium berghei, and to determine the extent to which the resulting attenuated parasites could induce a protective immunity in mice and rats. These studies were conducted toward the ultimate goal of achieving a better understanding of some of the mechanisms involved in acquired resistance to malaria.

MATERIALS AND METHODS

Male albino WRCF rats derived from the Wistar strain, weighing 40 to 50 g and male albino mice, Inbred Charles River Strain (ICR), weighing 20 to 25 g each, were used as test animals throughout these experiments. The NYU-2 strain of P. berghei, which had been maintained in the authors' laboratory for over three years in mice and two years in young rats, was used. No heterologous host cell materials or parasitic strains were employed.

The organisms used for immunization and infection were prepared as follows: blood was harvested from infected rats on the 14th day and from mice three or four days after inoculation with 2×10^7 P. berghei parasitized homologous RBC's. The number of parasitized RBC's per millilitre was determined by a vital staining technique described by Hillyer and Diggs [32].

For immunization, inoculum containing 2×10^8 red blood cells per millilitre was placed in each of a number of plastic tubes. The tubes were then placed in the irradiator and exposed to the desired amount of radiation; others were maintained under similar conditions for the duration of radiation and used as non-irradiated controls.

Control "immunizations" were made using saline citrate solution, irradiated normal rat blood and supernatant from irradiated parasitized inoculum. Normal blood dilutions were approximated by comparing haematocrits with the parasitized inocula and subsequent dilution. Irradiated inocula were centrifuged for 20 min at 2000 rev/min and the plasma was drawn off. This material was then inoculated through 13 mm 0.45 μm millipore swinney-13 filters. All control inocula were irradiated at 20 000 R and given intraperitoneally (IP) in a 1.5 ml volume for rats and a 0.5 ml volume for mice.

A Gammacell 220 cobalt-60 irradiator, described by Rice and Smythe [33], was used as the source of gamma radiation. This 1300 Ci source delivered a dose-rate calculated by the Fricke ferrous sulphate method of Weiss et al. [34] of approximately 900 R/min. The tubes containing the blood were placed in a circular rack. A planetary gear system permitted the test tube holder to rotate on its own while the test tubes were rotated on their axes. This method insured a uniform exposure to radiation of the red blood cells in each of the test tubes. The inoculum was kept in an ice bath until the time it was placed in the irradiator and afterwards until the animals were injected. All immunizations and challenges were given IP.

Animals were usually examined daily for the presence of malaria parasites which was determined by thick and thin blood smears stained with Giemsa stain. Parasite densities for rats were estimated as the number of infected cells per 50 fields. The total number of RBC's per field was estimated to be 100. Mouse parasitaemias were determined by counting the number of parasitized RBC's in 200 red blood cells. Sub-inoculations from mice and rats to mice were accomplished by diluting three drops of tail blood from each animal with 0.5 ml of saline citrate and injecting approximately 0.25 ml intravenously (IV) into two mice. The fluorescent antibody test using as antigen blood smears obtained from infected mice was performed as described by Diggs and Sadun [35]. The Wilcoxon Two-Sample Rank Test, Wilcoxon [36], White [37], was used to compare the averages of different groups.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

RESULTS

A first series of experiments was undertaken to determine the effect of exposure to gamma radiation on the ability of P. berghei to establish an

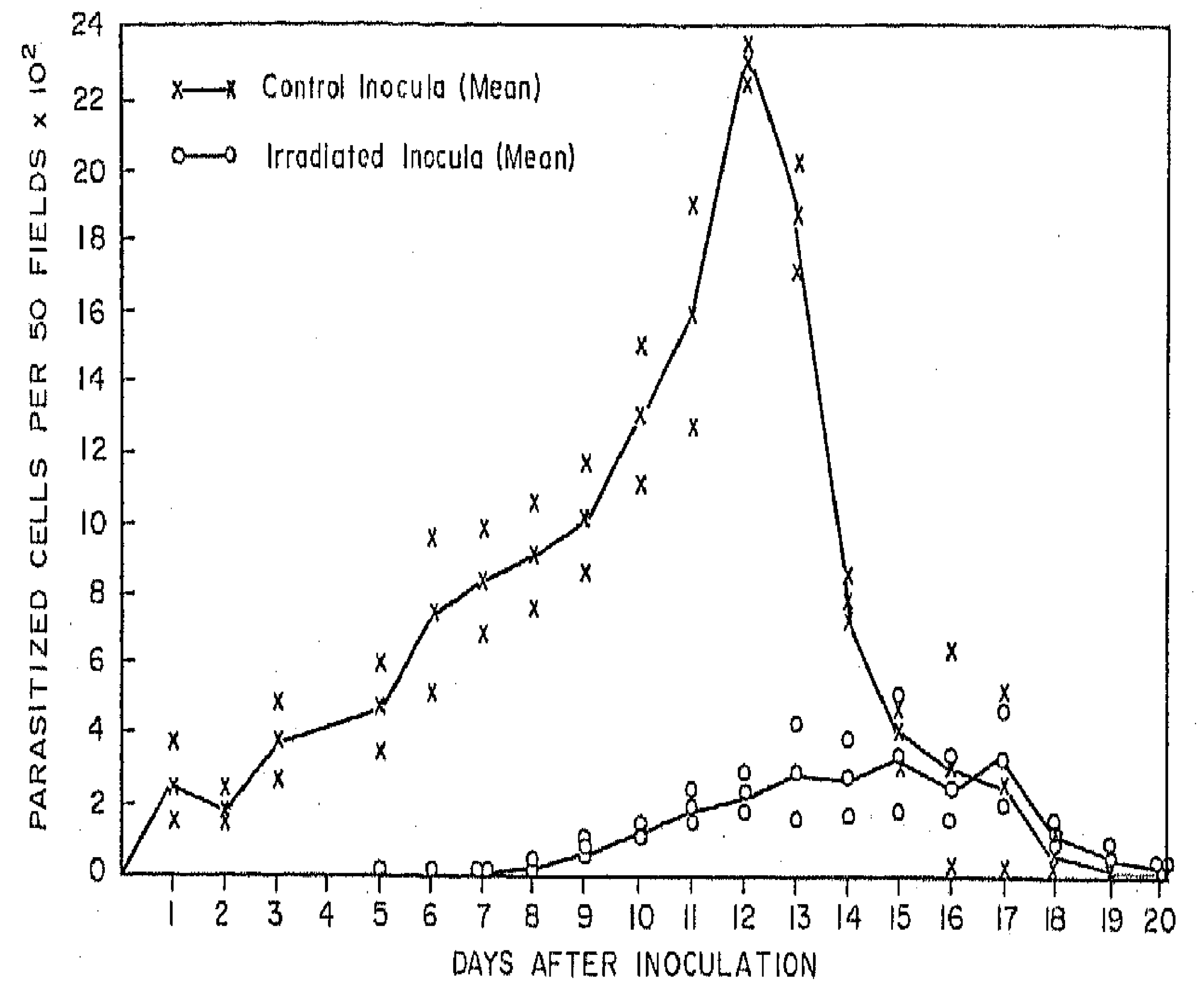

FIG.1. The course of parasitaemia in rats injected with 3×10^8 parasitized RBC's exposed to 16000 R.

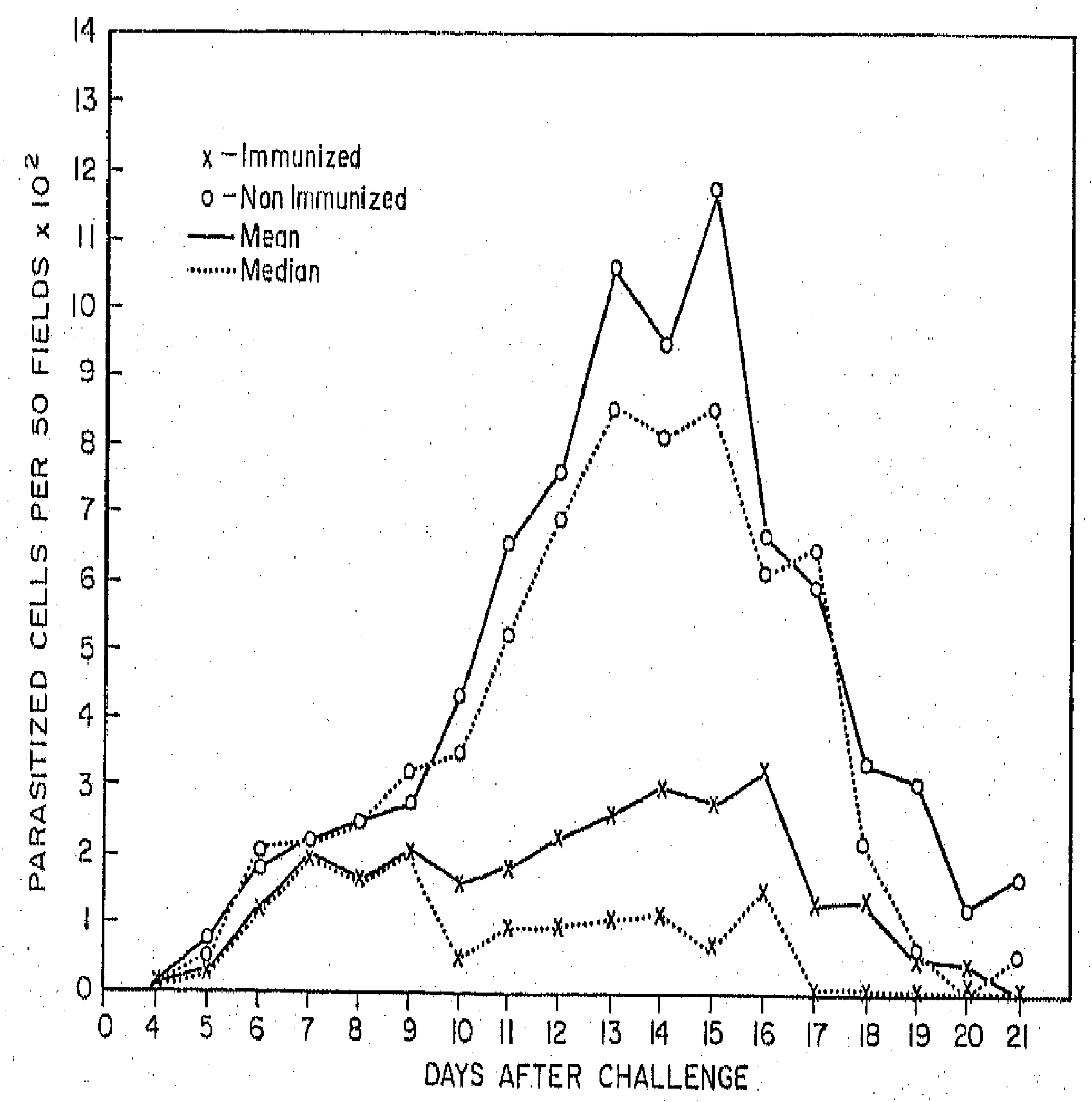

FIG.2. The course of parasitaemia in rats immunized once with 3×10^8 parasitized RBC's exposed to 20000 R, and challenged one week later with 2×10^4 parasitized RBC's.

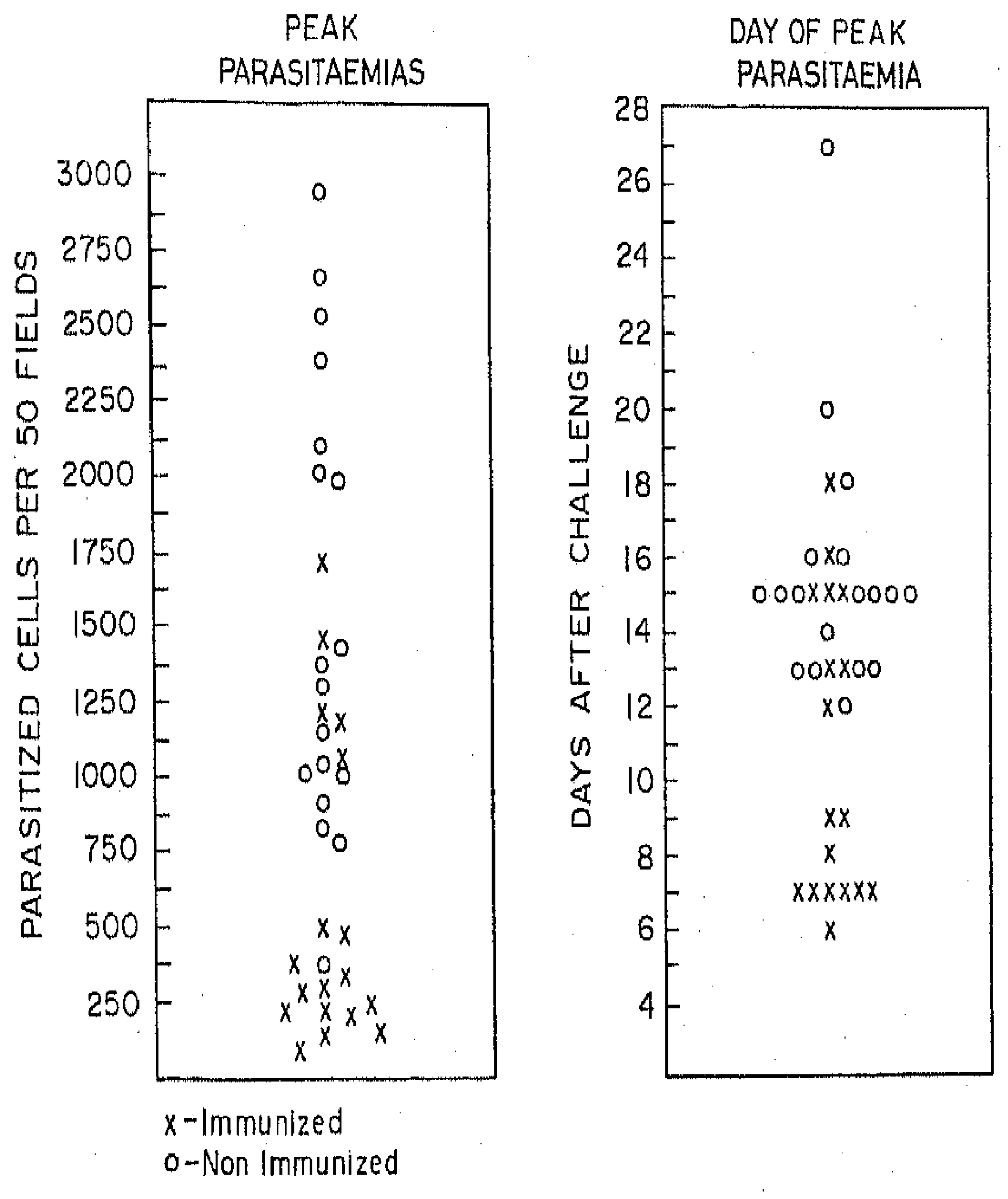

FIG.3. Level and time of peak parasitaemias in immunized and non-immunized rats (as in Fig.2).

infection in rats. A total of 115 rats was separated into 12 groups. The rats of the first group received parasitized RBC's exposed to 10000 R. Those of the other groups received approximately the same number of parasitized RBC's which had been exposed to different doses of radiation varying from 10000 to 60000 R. The results show that all animals infected with parasites exposed to doses up to and including 16000 R developed progressive parasitaemias with obvious parasite multiplication and re-invasion of RBC's. A certain number of rats receiving parasitized RBC's irradiated at levels of 17000 and 18000 R failed to develop progressive infections. With the exception of one of the rats inoculated with parasitized RBC's which had been exposed at 20000 R, infections were aborted by exposure to radiation at 19000 R or higher. The rats with aborted infections showed decreasing numbers of circulating parasites for two days and became negative on thick films usually by the third day following inoculation. The parasite cytoplasm in these cells when stained appeard pale blue and by the second day only nuclear material and pigment could be seen. Conversely, the non-irradiated control inocula produced an early patency with a rapidly ascending parasitaemia. Parasite multiplication and reinvasion of RBC's was observed in all animals receiving inocula irradiated at 16000 R. However, patent parasitaemias appeared considerably later and the number of parasitized RBC's increased at a much slower rate than in the controls receiving non-irradiated inocula (Fig.1).

In view of these results another series of experiments was undertaken to determine whether inoculation with parasitized blood exposed to 20000 R stimulated a demonstrable resistance to a challenge infection with non-irradiated parasites. As indicated in Fig.2, 36 rats were divided into two groups. Those of one group were inoculated with parasites irradiated at 20000 R and those of the other group served as controls. All the animals were challenged a week later with non-irradiated parasitized RBC's. On

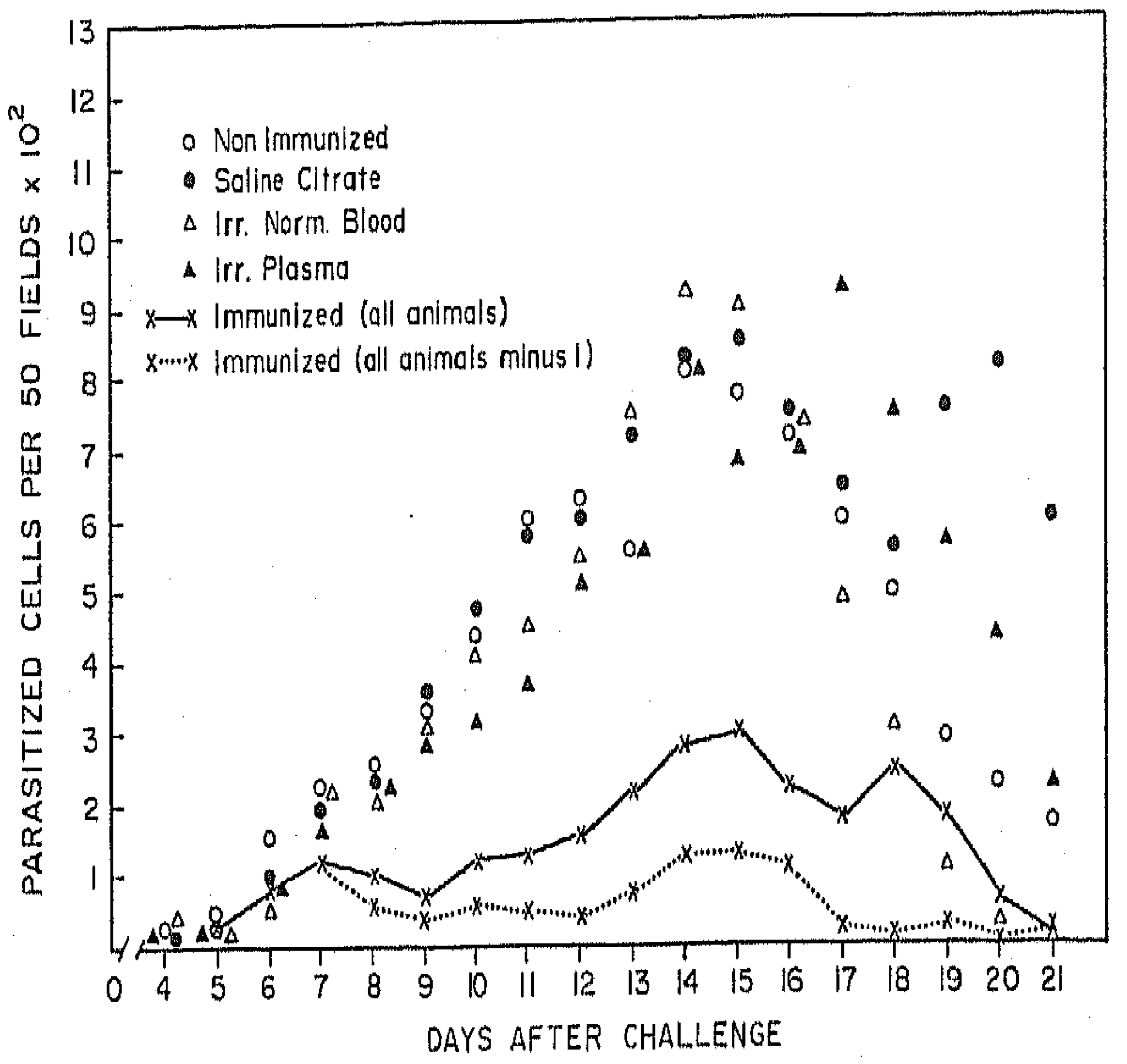

FIG.4. The course of parasitaemia in rats immunized with a single dose of 3×10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged one week later with 2×10^4 parasitized RBC's.

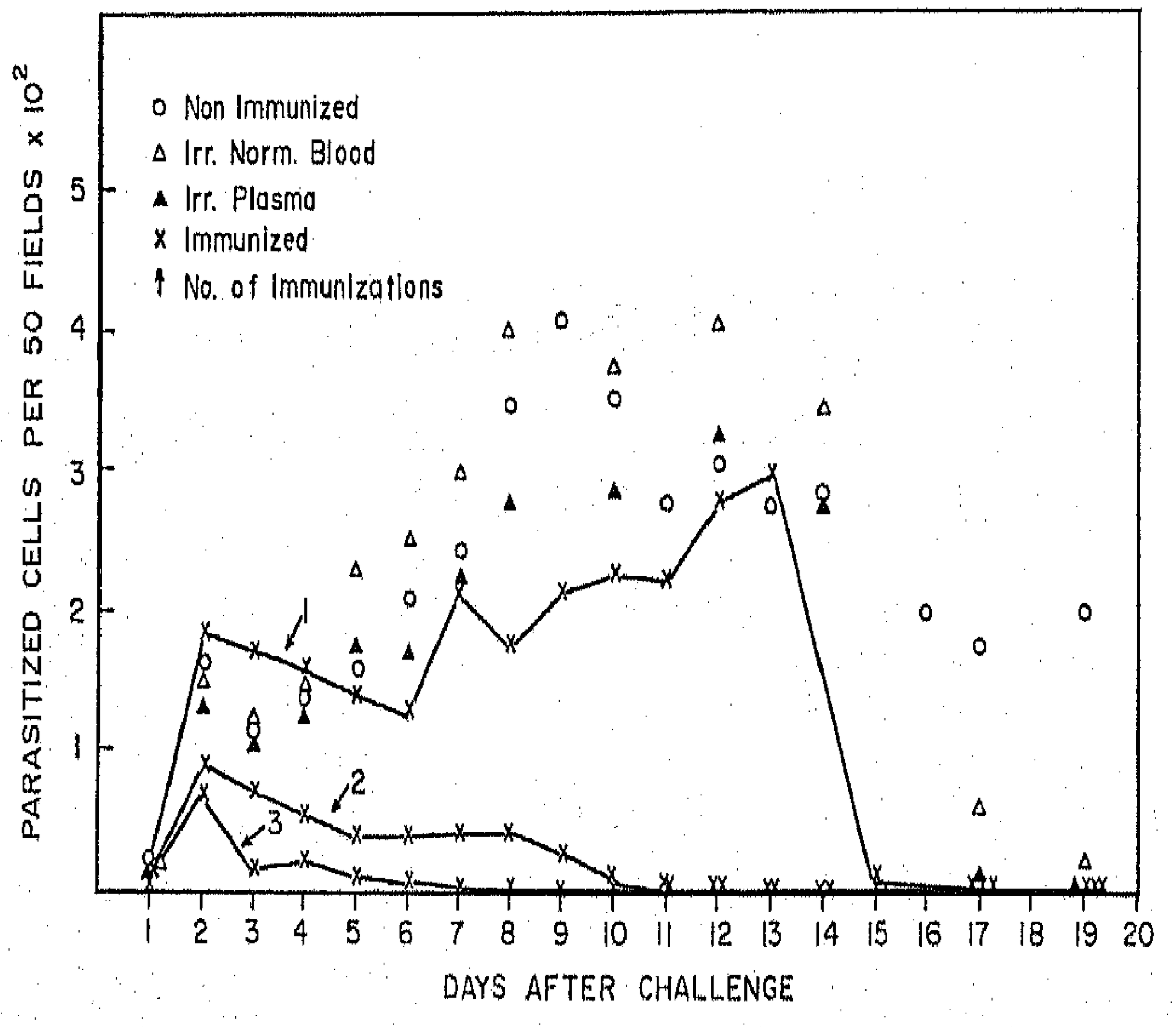

FIG.5. The course of parasitaemia in rats immunized by one, two, or three weekly doses of 3×10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged with 1×10^8 parasitized RBC's one week after the last immunization.

the day before challenge, blood from the rats which had received irradiated RBC's was subinoculated into normal mice to determine whether a subpatent parasitaemia might have resulted. No evidence of infection was discernible by repeated microscopic examination of thick blood smears obtained from these mice. After challenge with non-irradiated infected RBC's, the development of parasitaemia was compared in the two groups of rats (Fig. 2). Fewer parasites were present in the blood of the rats which had previously received irradiated parasites. A significant difference between the "immunized" rats and their controls was noticeable 10 days after challenge and persisted until after the crisis.

As indicated in Fig. 3, the peak parasitaemias reached in the immunized animals were significantly lower than those of the non-immunized controls and, with few exceptions, they fell into two distinct population groups. Likewise, reduction in parasitaemias began much earlier in the immunized rats than in their non-immunized controls. The difference between the two groups is highly significant ($P < 0.01$).

Since previous experiments, Martin et al. [10], had indicated that resistance to P. berghei could be induced non-specifically by substances other than of parasite origin, an additional experiment involving 30 rats was designed to determine whether the irradiated parasites per se were responsible in stimulating the observed acquired resistance. The rats were divided into five groups; those of one group received irradiated infected RBC's, others received irradiated plasma, irradiated non-infected RBC's or saline citrate solution. As indicated in Fig. 4, the animals that were previously inoculated with irradiated parasitized RBC's had lower parasitaemias following the challenging infection. If the one animal in the group which failed to develop a demonstrable acquired resistance is excluded from the series, highly significant differences between immunized and non-immunized groups were obtained ($P < 0.01$). No significant difference was observed among the animals in the four control groups (irradiated non-infected blood, irradiated saline citrate; plasma from irradiated parasitized blood and non-infected animals). No accurate comparisons could be made more than 17 days after challenge, since great variability of parasitaemia in individual observations occurred following the parasitic "crisis".

Two additional experiments involving 72 rats were designed to determine whether the degree of acquired resistance induced by inoculation with irradiated parasites was influenced by the number of immunizing exposures prior to the challenging infection. The results (Fig. 5) indicate that greater degrees of acquired resistance developed with increasing immunizing doses. The animals receiving three immunizing doses had a transitory parasitaemia resulting from the challenging infection.

All the control groups, each receiving three inoculations with irradiated non-infected blood, saline citrate, or plasma, developed a prolonged higher parasitaemia (normal course of infection). No significant difference was observed among the animals in the four control groups, and the haematocrit values of immunized rats remained between 40 and 50% after challenge with non-irradiated parasites, whereas the non-immunized controls developed a haemolytic anaemia (haematocrit: 25-30%).

In all of the preceding experiments immunizations were performed with parasitized RBC's exposed to 20 000 R. To determine whether the level of irradiation is critical in maintaining the ability of parasitized RBC's to induce an acquired immunity, another experiment was designed in which the infected RBC's were exposed to irradiation levels varying from 20 000 to 60 000 R. The results (Fig. 6) indicate that an acquired immunity developed following three inoculations with irradiated RBC's at all irradiation levels used.

In view of the encouraging results observed in rats, a series of experiments was set up to determine whether the acquired resistance, which is

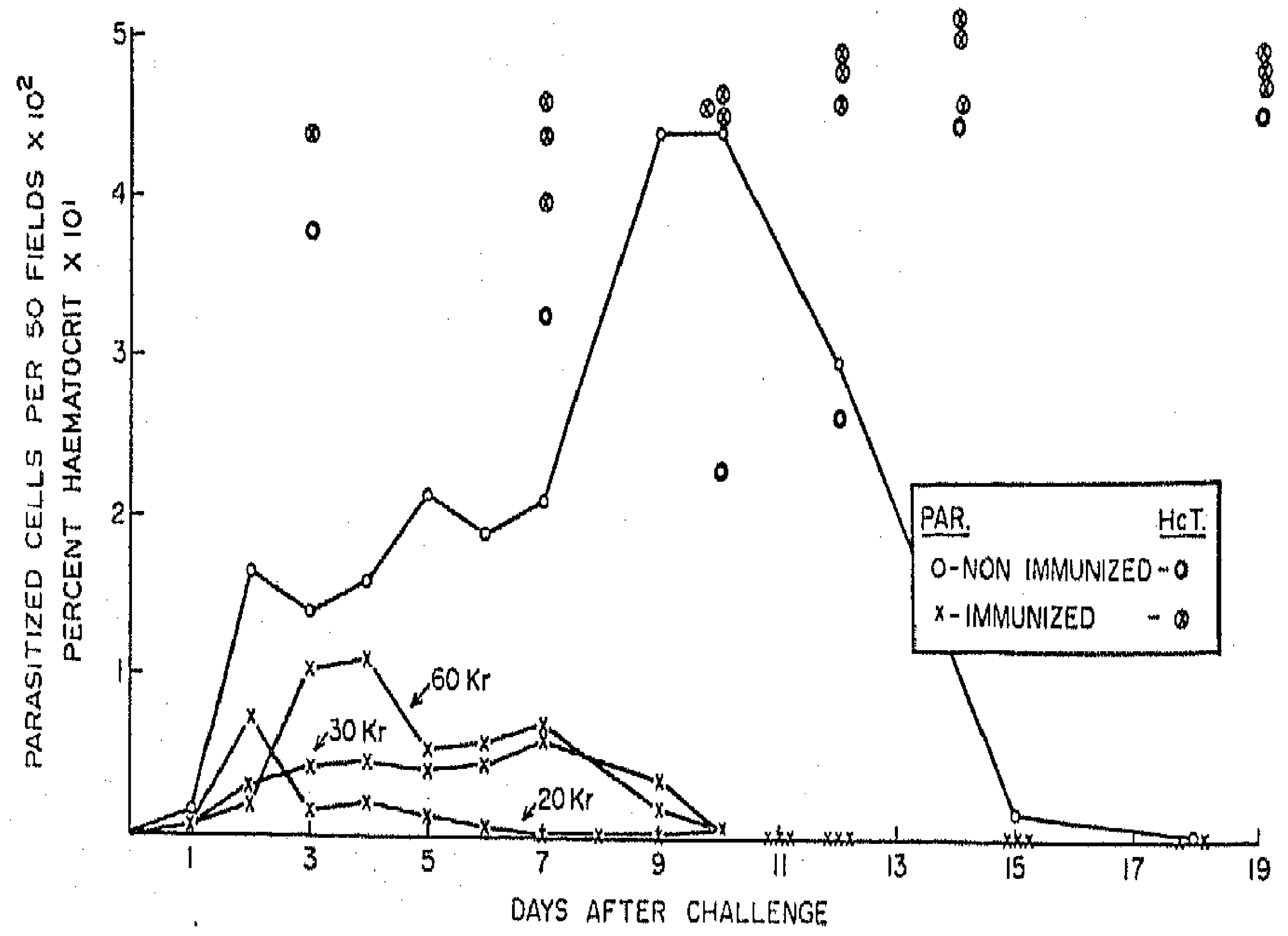

FIG. 6. The course of parasitaemia in rats immunized with three weekly doses of 3×10^8 parasitized RBC's exposed to 20 000, 30 000 and 60 000 R, respectively. All animals were challenged with 1×10^8 parasitized RBC's one week after the last immunization.

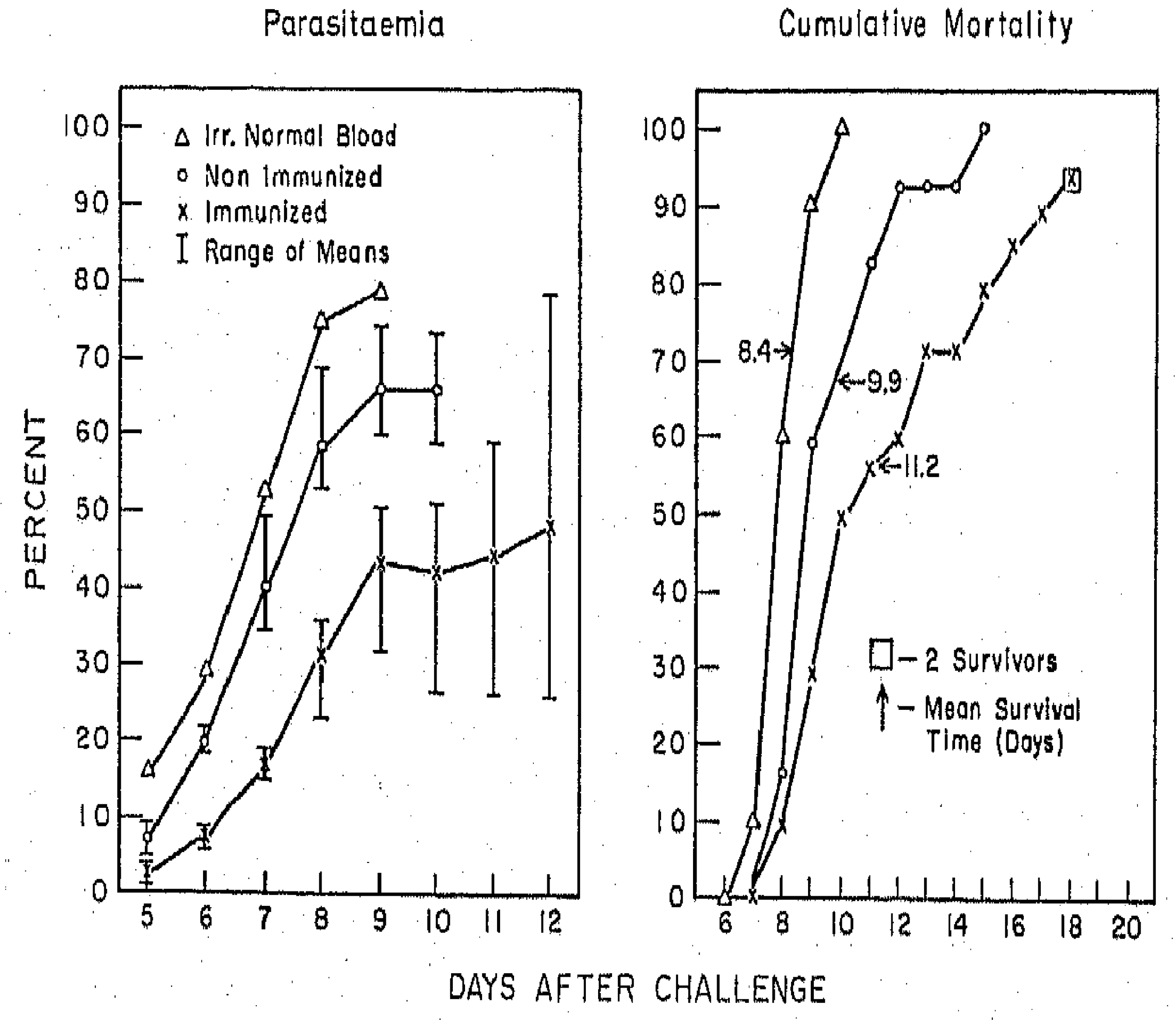

FIG. 7. Parasitaemia and cumulative mortality in mice immunized three times (twice weekly) by injections of 1×10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged with 2×10^4 parasitized RBC's one week after the last immunizing injection.

expressed in a marked reduction in the number of parasites developing from the challenging infection, could be detected also in a more susceptible animal, such as the mouse.

In three separate experiments a total of 70 mice was divided into three groups; those of the first group received three immunizing doses of irradiated infected RBC's; those of the second group served as non-immunized controls; and those of the third group received irradiated normal mouse blood. After challenge, one week following the last immunizing dose, the parasitaemia curves were compared. As shown in Fig. 7, lower per cent parasitaemia levels were observed in the immunized groups as compared with their controls. The cumulative mortality of the animals previously inoculated with irradiated parasites was also delayed (Fig. 7), and two of 30 immunized animals survived the challenging infection. One of these was negative by blood film throughout the experiment.

Additional experiments involving 120 mice were conducted to determine whether a greater series of immunizing inoculations would elicit a greater degree of acquired resistance. Groups of mice were immunized with five and ten doses instead of three. After the first immunizing inoculation parasitized red blood cells were visible for up to three days, but this was not so after subsequent immunizing inoculations. By the fifth immunization with irradiated RBC's parasites were no longer visible in thick blood films at any time. Mouse subinoculations after the first and fifth immunizing injections failed to produce a detectable parasitaemia in the recipient animals. The results (Figs 8 and 9) indicate that, with increasing immunizing doses, the level of parasitaemia resulting from the challenging inoculation was suppressed and that the parasitaemia developed at a much slower rate. Five of 35 mice receiving five immunizing doses survived while 6 out of 20 survived in the groups receiving 10 immunizing doses. At all three dose levels animals "immunized" with normal irradiated blood exhibited higher parasitaemias and shorter mean survival times than non-immunized control animals.

Two additional experiments involving 40 mice were designed to determine whether the degree of acquired resistance was influenced by the length of time between each immunizing inoculation and by the interval between the last immunization and the challenging infection. Mice were given five immunizing inoculations with irradiated plasmodia at weekly intervals. Some of the mice were challenged one week later, others two weeks later. The results (Figs 10 and 11) indicate that, while there were three survivors in the group challenged one week after the last immunizing dose, and no survivors in the group challenged one week later, a strong immunity was present in both groups. This immunity was expressed by slower progressing parasitaemias and much greater mean survival times.

To find out whether antibodies could be detected in mice which had been exposed to irradiated infected blood before challenge, the pooled sera were tested using the fluorescent antibody test. Positive results were obtained from these pools.

DISCUSSION

In view of the available epidemiological evidence on acquired immunity in parasitic infections, it is surprising that control by artificial immunization has proved to be so difficult. Due to the immunological complexities of the host-parasite relationship, attempts at immunization with vaccines prepared from dead parasites have given disappointing results when compared with immunity which can be stimulated by experimental infections. Thus it has been postulated that antigenic substances associated with living, actively metabolizing, parasites are important for producing a strong

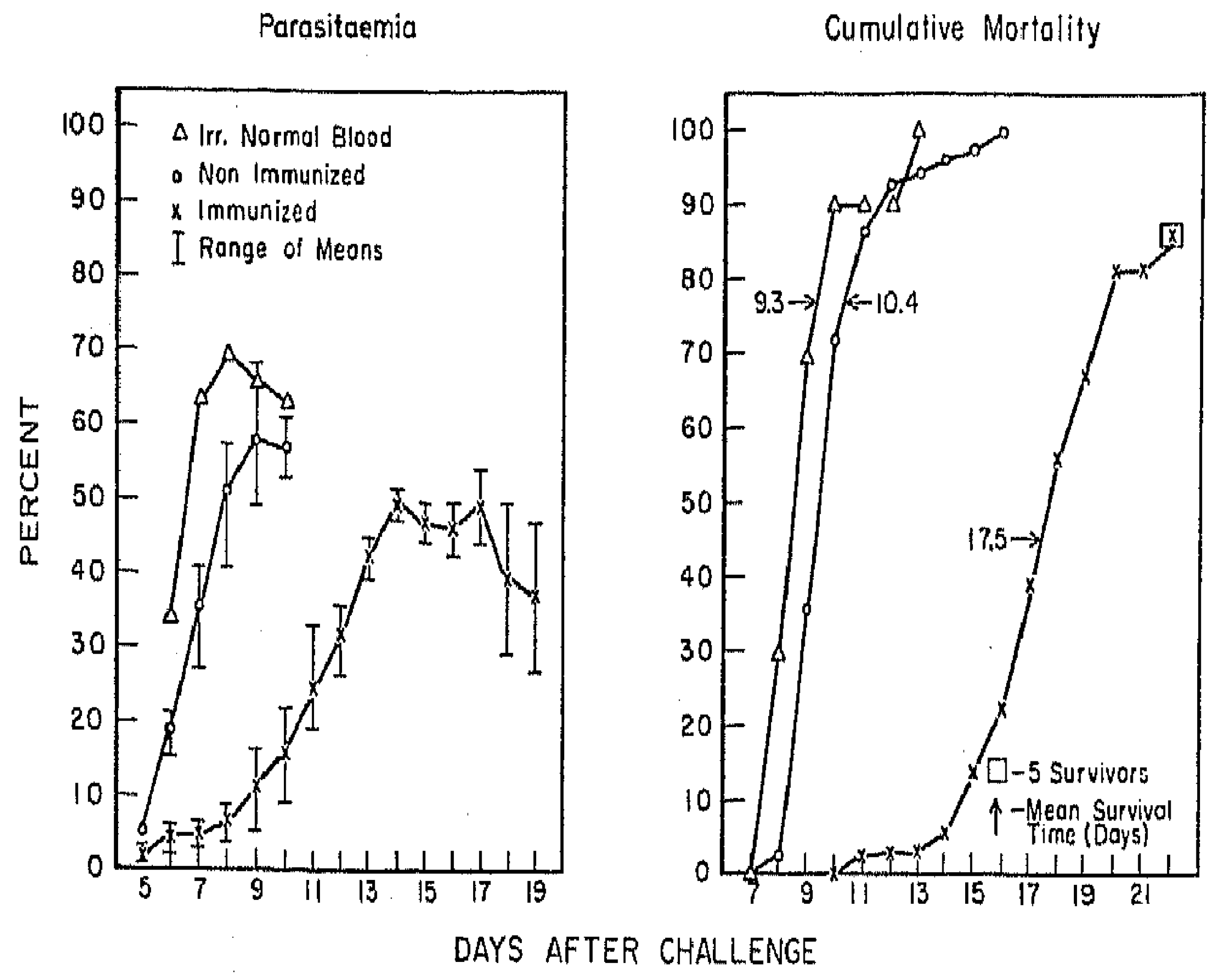

FIG. 8. Parasitaemia and cumulative mortality in mice immunized five times (twice weekly) by injections of 1 X 10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged with 2 X 10^4 parasitized RBC's one week after the last immunizing injection.

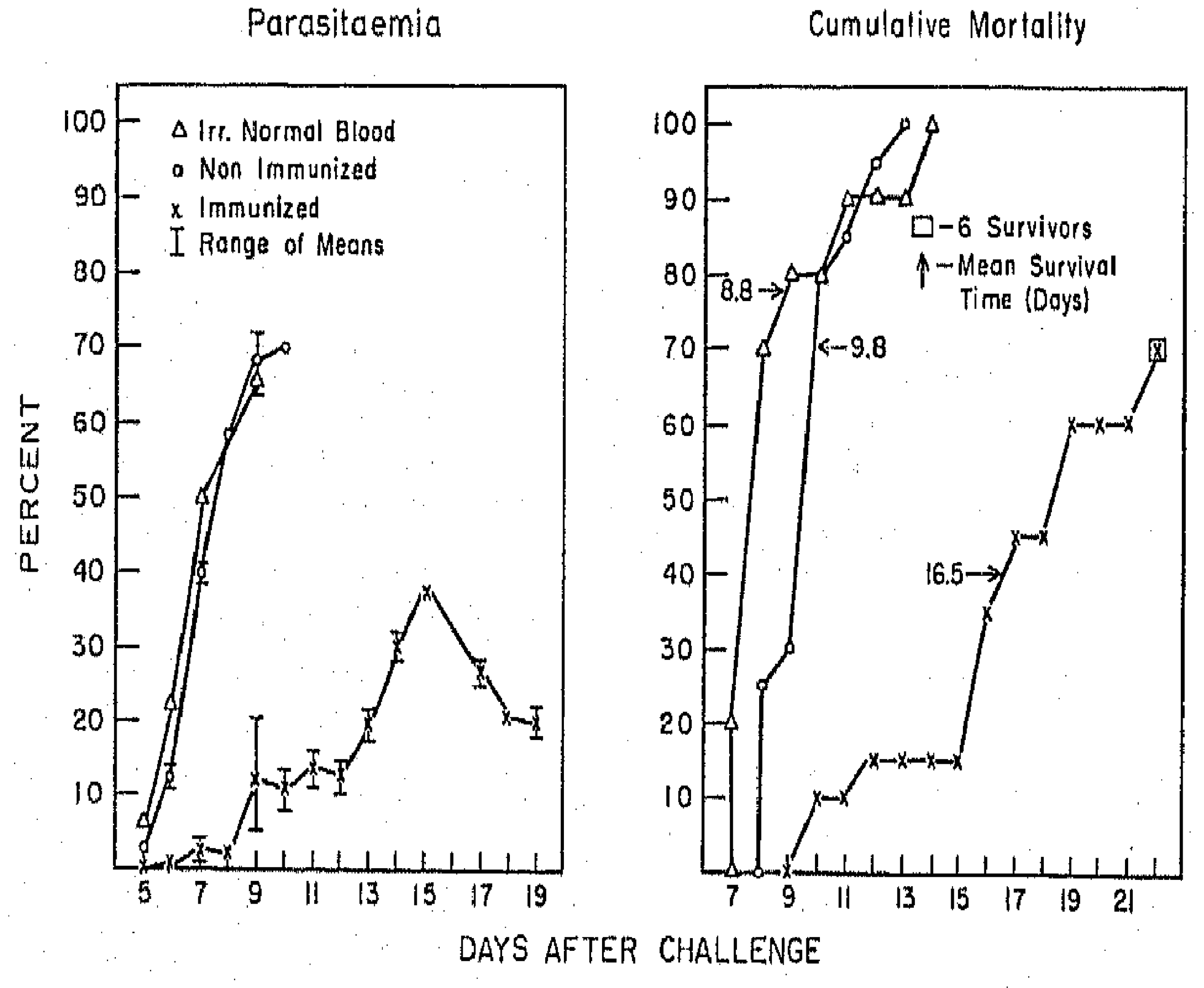

FIG. 9. Parasitaemia and cumulative mortality in mice immunized ten times (twice weekly) by injections of 1 X 10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged with 2 X 10^4 parasitized RBC's one week after the last immunizing injection.

immunity. Therefore, it is possible that vaccination with irradiated parasites may result in an acquired immunity as great as, or even greater than, that produced by infection with the living non-attenuated parasites, Miller [38].

An analysis of the foregoing experiments reveals, in general, that an acquired resistance to Plasmodium berghei is produced in rats and mice by vaccination with irradiated plasmodia. The resistance developed is sufficiently strong to reduce considerably the parasitaemia levels and the time of patency in the animals subjected to a challenging infection with non-irradiated parasites and to permit mice to survive otherwise lethal infections. This immunization is also sufficient to greatly minimize the damage resulting from the challenging infection, as evidenced by haematological observations and survival rates. These results confirm and extend those reported recently by Corradetti and his co-workers [31] in rats.

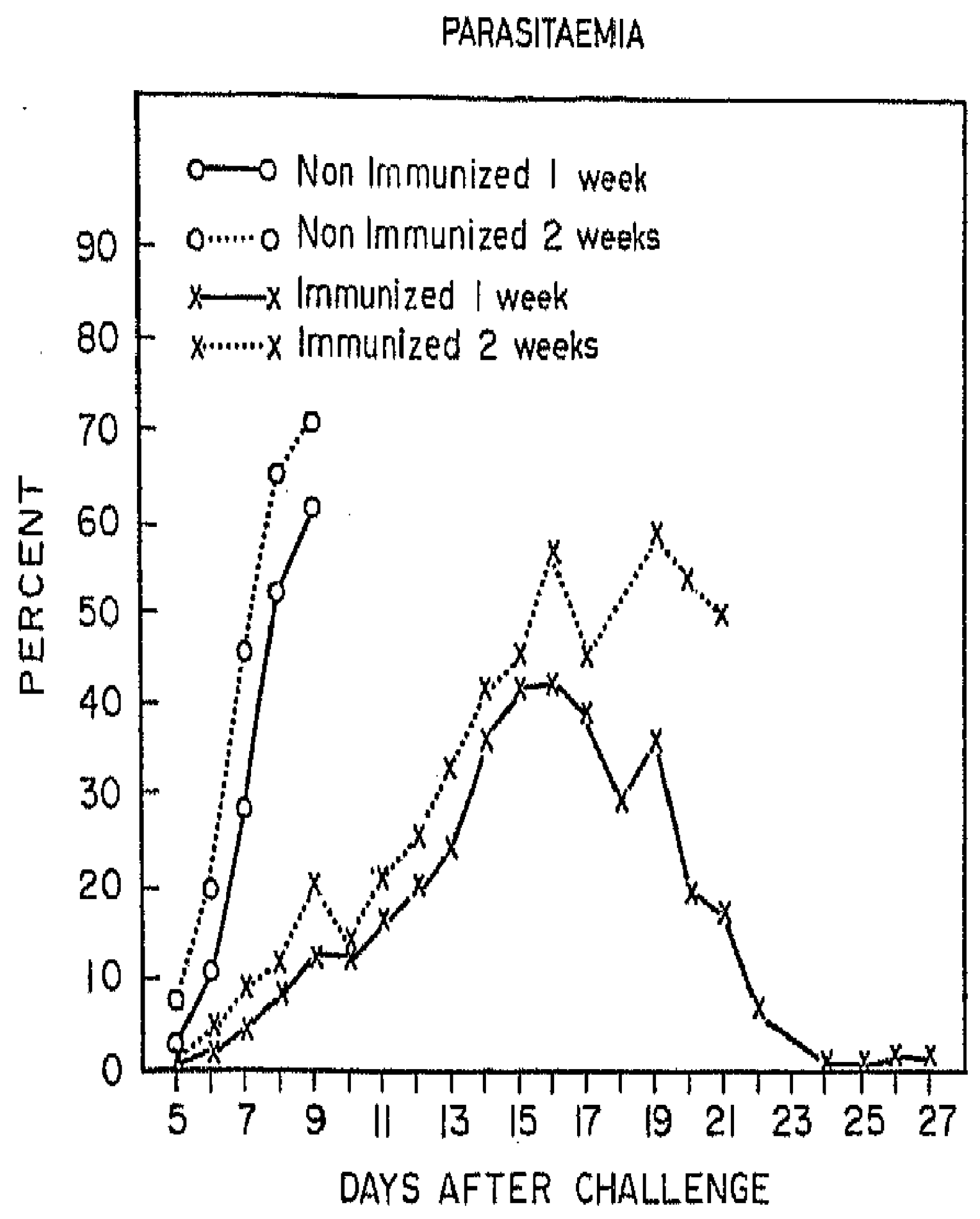

FIG. 10. The course of parasitaemia in mice immunized by five weekly injections of 1 X 10^8 parasitized RBC's exposed to 20 000 R. All animals were challenged one or two weeks after the last immunizing injection.

CUMULATIVE MORTALITY

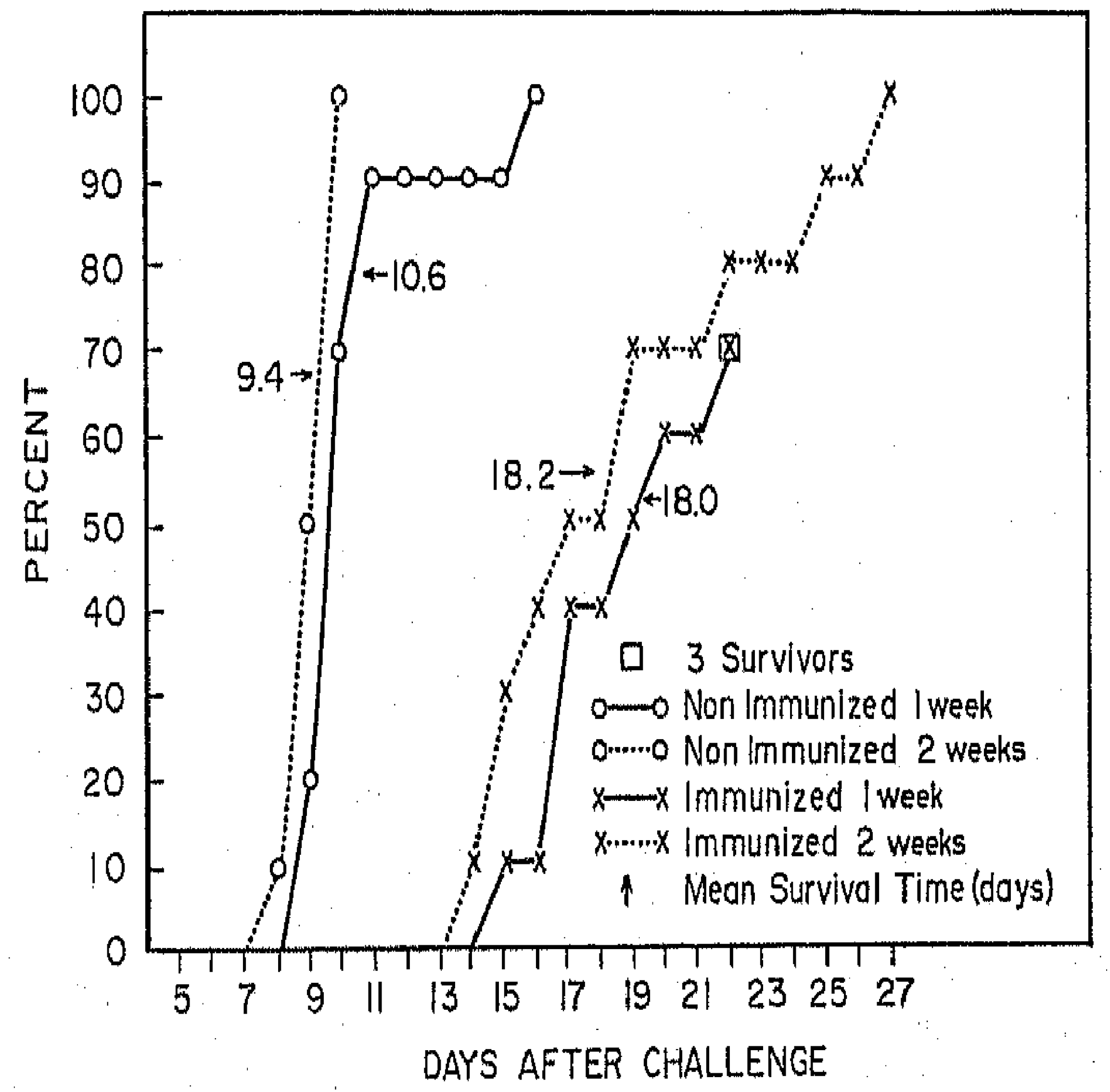

FIG. 11. Cumulative mortalities in mice (as in Fig. 10).

Autoimmunization has been accepted by many investigators as a working hypothesis to explain excessive anaemia in rodent malaria, Zuckerman [39], Cox et al. [40]. Although all of the observations described by these authors were compatible with an autoimmune mechanism of haemolysis, unequivocal proof is still lacking since none demonstrated the presence of erythrocyte auto-antibodies. The authors' results seem to indicate that in anaemia immunized by previous exposures to irradiated parasites, no marked anaemia developed after challenge with non-irradiated parasites. These results are in agreement with the findings reported by George and his co-workers [41].

It must be emphasized that even the greatest acquired immunity observed among the animals receiving multiple vaccinations with irradiated parasites was not always absolute. Both in rats and mice the degree of acquired immunity was directly proportional to the number of immunizing doses. Although no systematic studies were conducted to determine the optimal time interval between immunizing doses and between immunization and challenge, similar results were obtained when immunizations occurred at weekly intervals or when given twice weekly. Likewise, significant differences were not detected when the challenge took place one or two weeks after the last immunizing dose.

The results of experiments conducted in mice indicating that vaccination with irradiated parasites induces a resistance sufficient to protect them from an otherwise lethal infection is indeed noteworthy. To the authors' knowledge, this is the first time that a significant number of mice has been able to interrupt the active multiplication of an established infection without the administration of chemotherapeutic agents. These results suggest the possibility of using percentage mortality figures as a sensitive and practical additional tool for the determination of acquired resistance to *P. berghei* in mice. Furthermore, vaccination with irradiated plasmodia may be used for the production of sufficiently large numbers of "immune" mice for studies on the nature of the mechanisms of immunity to *P. berghei* in a susceptible host.

The observation that in nearly all of the mice which were not completely protected by vaccination with irradiated parasites the parasitaemia rose at a much slower rate than that of the controls is of equal interest. These results contrast with those obtained in splenectomized chimpanzees infected with *P. falciparum*, Sadun, et al. [42], and in mice infected with *P. berghei*, Briggs et al. [9], following injection of immune serum and indicate that the apparent qualitative difference among the antigenic components found in parasites during successive relapses (relapse variants) may be primarily due to differences in the relative proportions of common antigens. This possibility is strengthened by the observation that a low-grade parasitaemia resulting from the challenging dose in a few "vaccinated" mice persisted over a long period of time prior to the development of a sterilizing immunity. Evidence that mice with chronic infections may undergo spontaneous cure has been presented by Cox [43] and Garza and Box [44].

The results obtained in the authors' studies designed to determine the minimum radiation doses which would reduce significantly the survival and multiplication of plasmodia indicate that progressive infections were prevented, except in one instance when the parasites were exposed to 19 000 R or more. This irradiation level is essentially the same as that reported previously by Bennison and Coatney [13], and Corradetti et al. [31]. Subinoculations into susceptible mice from parasites thus treated failed to produce active infections. Yet, the fact that vaccination with parasites irradiated at these or higher levels was effective both in mice and rats provides additional evidence in support of the view that the presence of replicating plasmodia in the host is not necessary for the development of a strong degree of acquired resistance. This is in agreement with the concept of "sterile immunity" described for rats by Corradetti and his co-workers [1] or true residual immunity described in treated mice, Box and Gingrich [4], Briggs et al. [6], and at variance with the concept of premunition, Sergent [45].

It was of interest for theoretical and diagnostic considerations to determine whether antibodies are produced in sufficient amounts in the vaccinated animals to be detected by the fluorescent antibody test. The results indicated that mice and rats were able to produce a detectable level of antibodies even when the normal development and multiplication of plasmodia was interrupted by irradiation. These results are in agreement with and extend those reported from animals injected with irradiated *Trichinella spiralis* larvae, Hendricks [46], Sadun et al. [47], or with irradiated *Schistosoma mansoni* cercariae, Sadun et al. [48].

In view of the promising results obtained with the induction of acquired resistance to *P. berghei* in rats and mice by previous vaccination with irradiated plasmodia, similar studies have been initiated with *P. knowlesi* and *P. falciparum* infections. The relative role of humoral and cellular factors in inducing immunity following vaccination with irradiated plasmodia is also being investigated.

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IMMUNE MECHANISMS IN MICE IMMUNIZED WITH IRRADIATED PLASMODIUM BERGHEI

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(Short communication)

Acquired resistance to P. berghei has been observed in mice inoculated with irradiated parasites. The studies described below were done to elucidate some of the cellular and humoral mechanisms involved.

Mice were immunized with five inoculations of irradiated P. berghei and challenged with non-irradiated parasites one week after the last immunization. The parasitaemias of control animals rose rapidly, whereas parasitaemias of immunized mice progressed at a much slower rate.

On the day of challenge a significant increase in gamma globulins was noted in the immunized mice. Serum collected at this time contained protective antibodies as determined by passive transfer tests. Since parasitaemias persisted in the challenged mice it seemed possible that such parasites represent variant or derived strain(s) relatively unsusceptible to cellular and humoral factors of acquired immunity. To compare antiserum susceptibility of parasites from control mice (stock strain parasites) with that of parasites from experimental mice (suspected variant strain parasites), subinoculations of parasites were made from both groups and parasites in recipient mice were then exposed to mouse antiserum. Antiserum collected on the day of challenge showed a relatively marked effect against the parasites of the stock strain but was relatively ineffective against parasites of the derived or variant strain. Rat antiserum from other sources showed similar differences in effectiveness against parasites of these two strains, providing further evidence for immunological differences in stock and derived strains of parasites. Mean-spleen weights of immunized mice were double the weights of the controls.

IMMUNE RESPONSE OF CATTLE INFECTED WITH TRYPANOSOMA BRUCEI SUBGROUP AND THE ANTIGENIC VARIATION OF INFECTING ORGANISMS

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Abstract

IMMUNE RESPONSE OF CATTLE INFECTED WITH TRYPANOSOMA BRUCEI SUBGROUP AND THE ANTIGENIC VARIATION OF INFECTING ORGANISMS. During T. brucei group infection in cattle a succession of antigenic variant trypanosome populations develop. In response to these antigenic variants a succession of agglutinating and neutralizing antibodies are produced, which remain detectable for extended periods and probably preclude the reappearance of a particular antigenic variant. These antigenic variant populations also have antigens in common with each other, and with the infecting stabilate, which stimulate the production of antibodies detectable by precipitation and fluorescent antibody techniques. It is considered probable that the reaction between common antigens and homologous antibodies may play a part in the pathogenesis of the disease.

The antigenic lability of organisms of the Trypanosoma brucei subgroup during infection in laboratory animals has been widely investigated over the last 60 years. The literature on the subject has recently been reviewed by Gray [1] who himself investigated the phenomenon in rabbits infected with T. brucei.

In this report the antigenic variation of organisms of the T. brucei group during infection in cattle, and the antibody response of these cattle, have been studied.

MATERIALS

Cattle

East African shorthorned Zebu from the East African Trypanosomiasis Research Organization (EATRO) herd, described by Smith [2] were used. The five cattle in this experiment were free from trypanosomiasis as judged by serological tests, inoculation of blood into mice, and examination of stained blood films. Four of the cattle were bullocks (Nos 5, 480, 616 and 840) and one was a cow (No. 612). At the beginning of the experiment the cattle were between two-and-a-half and three years of age. During the experiment the cattle were grazed in a tsetse-free area and received no supplementary feeding.

Trypanosomes

The trypanosomes were the Trypanosoma brucei Plimmer and Bradford Subgroup, Hoare [3], originally isolated in mice from a blood lake, Cockings [4], fed on by about 100 Glossina pallidipes Austen which were caught at Lugala, Busoga District, Uganda in July 1960. The trypanosomes were preserved at approximately -80°C, Cunningham, Lumsden and Webber [5], on the second day of the third passage in mice and designated stabilate, Lumsden and Hardy [6], EATRO-344. Stabilates EATRO-2, EATRO-3 and EATRO-691 are all derivatives of EATRO-344.

Mice

Swiss white mice from the EATRO colony were used. This stock originated at Carworth Farms, New York and was brought to Africa in 1939, Smithburn and Haddow [7].

Diluent for serological tests

The diluent used was BS-2, a borate buffered salt solution, pH-8.0, as described by Lumsden, Cunningham, Webber, van Hoeve and Walker [8].

METHODS

Measurement of infectivity of trypanosome suspensions

The suspension containing trypanosomes was serially diluted tenfold in BS-2, and 0.1-ml aliquots of each dilution were inoculated into groups of six mice. From the number of mice in each group which became parasitaemic, a 63% end point was calculated (ID_{63}), which is directly comparable (assuming that one trypanosome can infect a mouse) with visual counts of organisms, as obtained by using a haemocytometer. The method is fully described by Lumsden et al. [8].

Inoculation of cattle and collection of samples

Four cattle (Nos 480, 612, 616 and 840) were inoculated intravenously with $16^{6.5}$ mouse ID_{63}'s of EATRO-3. Stained blood films were examined daily, except Sundays, for 80 days. Serum was obtained at regular intervals and stored at approximately -20°C until required. Blood from the cattle was inoculated into mice at weekly intervals for 12 weeks and thereafter at four-week intervals for a year. The isolates so obtained, up to and including the 20-week isolate, were passaged in mice (maximum five passages) at less than three-day intervals until parasitaemias of the order of 10^8 trypanosomes per millilitre were obtained, and were then preserved alive in lymph tubes at approximately -80°C, Cunningham et al. [5], for use as antigens in the serological tests.

Bullock No. 5 was inoculated intravenously with 10^6 mouse ID_{63} of EATRO-691. Heparinized blood samples (four units heparin per millilitre blood) were collected from the cow at daily intervals for 17 days. The infectivity of the daily samples was measured by serial tenfold dilution and inoculation into groups of mice. Blood, containing trypanosomes, was preserved daily at approximately -80°C. Serum samples were obtained and stored at approximately -20°C until required. The daily serum samples were used in a series of neutralization tests against EATRO-691 and against the stabilates preserved on the 7th and 13th days after infection.

Bullocks Nos 23, 167, and 985 were used to investigate the antigenic identity of three stabilates isolated from Bullock 480, one, five and nine weeks respectively after infection with EATRO-3. These stabilates were designated 480/1, Bullock 167 with 480/5 and Bullock 985 with 480/9. In each case, the infecting dose was approximately 10^8 mouse ID_{63}. Three days after inoculation of the organisms the cattle were treated with Berenil (Farbwerke Hoechst AG, Frankfurt, Federal Republic of Germany) intramuscularly, at a dose-rate of 7 mg/kg body weight. Serum was obtained from the cattle at regular intervals during one month and stored at approximately -20°C. These sera were used to investigate the agglutinin and neutralizing antibody response of the cattle to the inoculated stabilates. Using the agar-gel double diffusion test, antigens from each stabilate were tested against serum samples from cattle Nos 480, 612, 616 and 840, in an attempt to demonstrate common antigens.

Bullock No. 59 was inoculated intravenously with approximately 10^8 ID_{63} of each of the stabilates 480/1, 480/5 and 480/9. Three days after inoculation of the organisms it was treated with Berenil as above. Serum was obtained at regular intervals during one month, stored at approximately -20°C, and used to examine the agglutinin and neutralizing antibody response.

Serological tests

Four serological tests used were as follows:

(a) Agglutination test

The test described by Cunningham and Vickerman [9] was used.

(b) Neutralization test

This test may be qualitative or quantitative.

Qualitative. To examine serum for the presence or absence of neutralizing antibodies, approximately $10^3 ID_{63}$ of the relevant trypanosome stabilate was incubated in one millilitre of undiluted control and test serum samples for 30 min at 0°C. Groups of six mice were then inoculated with 0.1-ml aliquots of each suspension. Neutralizing antibodies were assumed to be present in those samples which completely inhibited infection in mice, as compared with the control sample.

Quantitative. The relevant trypanosome stabilate was incubated in a suitable dilution (a dilution as determined by the qualitative method, which incompletely inhibits infectivity in a group of mice) of control and test serum for 30 min at 0°C. Using the infectivity titration technique, Lumsden et al. [8], the number of ID_{63}'s for mice in each suspension was then determined. The anti-trypanosomal activity of the test serum, as compared with the control serum, is expressed as the Neutralizing Index, which is the $\log_{10}$ of the number of ID_{63}'s inhibited by one millilitre of undiluted test serum.

(c) Agar-gel double diffusion test

A 1% solution of filtered Difco-Bacto-Agar in hot isotonic saline, with 1/10 000 merthiolate was poured into Petri dishes to a depth of 3 to 4 mm. Wells were cut 1 cm in diameter and 4 mm apart. Whole trypanosomes used as antigen were obtained from heparinized rat blood within three days of inoculation of the rats with the relevant trypanosome stabilate. The trypanosomes were concentrated and washed free of rat blood components by differential centrifugation at 0°C, using BS-2 as diluent. Centrifugation at higher temperatures in unbuffered diluent causes disruption of some of the organisms, with consequent loss of antigenic material in the supernate (for the effect of pH on trypanosomes, see Lumsden, Cunningham, Webber, Knight, van Hoeve and Simmons [10]. Usually the trypanosome concentrate was used immediately, but it was occasionally preserved at approximately -80°C before use.

(d) Indirect fluorescent antibody test

This was an adaptation of the test described by Kimber [11] for the demonstration of antibodies against trypanosomes in human serum obtained from sleeping sickness cases.

For use as antigen trypanosomes in heparinized blood from infected rats or mice with a rising parasitaemia were concentrated by centrifugation at 0°C, separated from RBC's and smeared on 3-in X 1-in glass slides. When dry the films were fixed by gentle heat and the slides sealed in a container which was stored at approximately -20°C until required. Before use ten circles of clear nail varnish, each approximately 0.5 cm in diameter, were applied to the slides, using a syringe fitted with a blunted 24-gauge needle. To carry out the test 0.025 ml volumes of two-fold dilutions of test serum in phosphate buffered saline (PBS), pH 7.4, were placed within the circles of varnish, and incubated at 25°C for one hour in a moist atmosphere, after which slides were washed in three changes of PBS during a period of 20 min and then blotted dry with filter paper. A suitable dilution of anti-bovine globulin conjugate (FA-Bovine Globulin Antiglobulin (Rabbit), Difco Laboratories Detroit, Michigan) in PBS was then applied within each circle of varnish in 0.025-ml volumes. After incubation in a moist atmosphere for 40 min at 25°C the slides were washed in three changes of PBS, blotted dry and mounted in glycerol buffered to pH 8.0 under No. 0 cover slips. In each batch of tests known positive and negative controls were included and the conjugate was tested for non-specific fluorescence.

The slides were examined under the X 40 objective of a "Conference" microscope (Gillett and Sibert Ltd., London) using a 100 W, 12 V iodine quartz lamp as a light source. The light was passed through two primary filters (No. 30/063), and one secondary filter (No. 10/285). Fluorescent trypanosomes stand out as apple green against a brown background.

Results obtained from investigations concerning cattle Nos 480, 612, 616 and 840

The four cattle inoculated with EATRO-3 all became parasitaemic. Nos 480, 612 and 840 were parasitaemic by the seventh day as judged by the inoculation of blood into mice, and continued so until between 8 and 10 months after infection. Blood from Bullock No. 616 collected seven days after inoculation of EATRO-3 did not cause infection in mice, but the 14-day sample, and all subsequent samples up to a year later, consistently infected mice. Trypanosomes were present on stained blood films from all cattle on a few occasions within the first three weeks after infection and were then consistently absent until examination ceased 80 days after infection. The cattle remained in apparent good health throughout the course of the experiment.

(a) Results obtained using the agglutination test

Using EATRO-3 as antigen, agglutinins were detected as early as the sixth day after infection, reached a peak during the second or third week, fell sharply, and then continued to fall gradually. Agglutinins were still detectable in sera collected 250 days after infection (see Fig. 1).

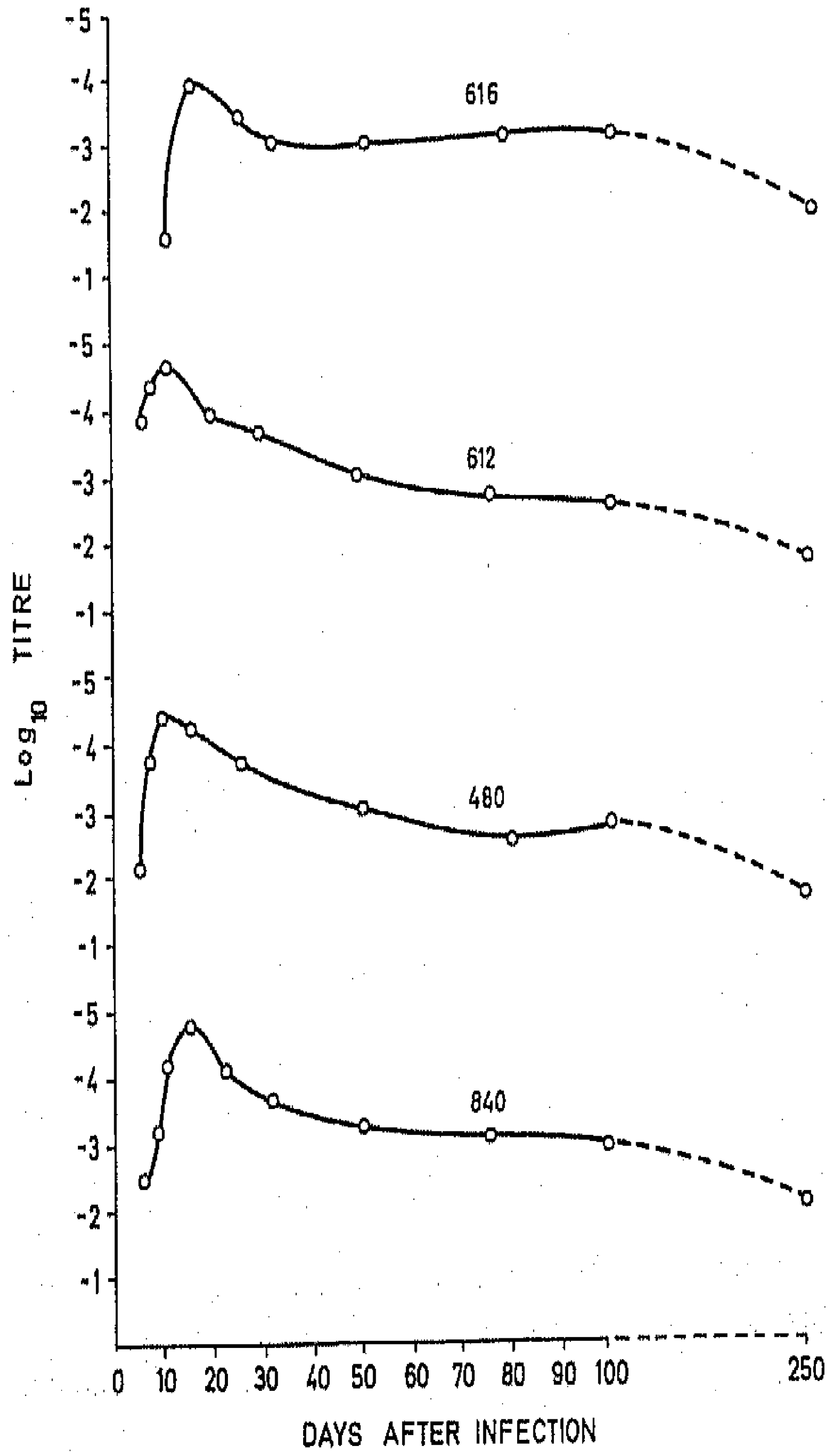

FIG. 1. Serum agglutinin titres against EATRO-3.

Using the serial isolates as antigen, agglutinins were not generally found in sera collected prior to, or at the same time as, the isolation of the organisms. Positive results were obtained, however, with most sera collected subsequent to the isolation of the organisms. Agglutinins against all of the isolates remained detectable for extended periods. The results are shown in Tables I-IV.

TABLE I. AGGLUTINATION RESULTS USING SERUM AND TRYPANOSOME ISOLATES, OBTAINED FROM BULLOCK No. 480

		Trypanosome stabilates												
	Day[x]	7	14	21	28	35	42	49	56	63	70	84	112	140
SERUM	32	+	+	+	+	0	0	+	+	+	0	0	+	+
	60	+	+	+	+	+	+	+	+	+	0	0	+	+
	88	+	+	+	+	+	+	+	+	+	+	+	0	0
	116	+	+	+	+	+	+	+	+	+	+	+	+	0
	144	+	+	+	+	+	+	+	+	+	+	+	+	0

+ Positive agglutination or neutralization.
0 Negative agglutination or neutralization.
[x] Figures refer to the day of infection on which serum was collected or trypanosomes were isolated.

TABLE II. AGGLUTINATION RESULTS USING SERUM AND TRYPANOSOME ISOLATES OBTAINED FROM COW No. 612

		Trypanosome stabilates													
	Day[x]	7	14	21	28	35	42	49	56	63	70	77	84	112	140
SERUM	32	+	+	+	+	0	0	+	0	0	0	0	0	0	0
	60	+	+	+	+	+	+	+	+	0	0	0	0	0	0
	88	+	+	+	+	+	+	+	+	+	+	+	+	0	0
	116	+	+	+	+	+	+	+	+	+	+	+	+	+	0
	144	+	+	+	+	+	+	+	+	+	+	+	+	+	0

+ Positive agglutination or neutralization.
0 Negative agglutination or neutralization.
[x] Figures refer to the day of infection on which serum was collected or trypanosomes were isolated.

(b) Results obtained using the neutralization test

The neutralization test was applied only to material collected from Bullock No. 840. Using EATRO-3 as antigen, neutralizing antibodies were detectable by the sixth day after infection, reached a peak during the second week, and continued to fall gradually until the 226th day after infection (see Fig. 2).

TABLE III. AGGLUTINATION RESULTS USING SERUM AND TRYPANOSOME ISOLATES OBTAINED FROM BULLOCK No. 616

		Trypanosome stabilates											
	Day[x]	14	21	28	35	42	49	56	63	70	77	84	112	140
SERUM	32	+	+	+	0	0	0	0	0	0	0	0	0	0
	60	+	+	+	+	+	+	+	0	0	0	0	0	0
	88	+	+	+	+	+	+	+	+	+	+	+	0	0
	116	+	+	+	+	+	+	+	+	+	+	+	+	0
	144	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Positive agglutination or neutralization.
0 Negative agglutination or neutralization.
[x] Figures refer to the day of infection on which serum was collected or trypanosomes were isolated.

TABLE IV. AGGLUTINATION RESULTS USING SERUM AND TRYPANOSOME ISOLATES OBTAINED FROM BULLOCK No. 840

		Trypanosome stabilates													
	Day[x]	7	14	21	28	35	42	49	56	63	70	77	84	112	140
SERUM	32	+	+	+	+	0	0	0	0	0	0	0	0	0	0
	60	+	+	+	+	+	+	+	+	0	0	0	0	0	0
	88	+	+	+	+	+	+	+	+	+	+	+	+	+	0
	116	+	+	+	+	+	+	+	+	+	+	+	+	+	0
	144	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ Positive agglutination or neutralization.
0 Negative agglutination or neutralization.
[x] Figures refer to the day of infection on which serum was collected or trypanosomes were isolated.

Using three of the serial isolates as antigen, neutralization was observed only with sera collected subsequent to the isolation of the organisms (see Table V). Neutralizing antibodies against each of these isolates were still detectable 226 days after infection.

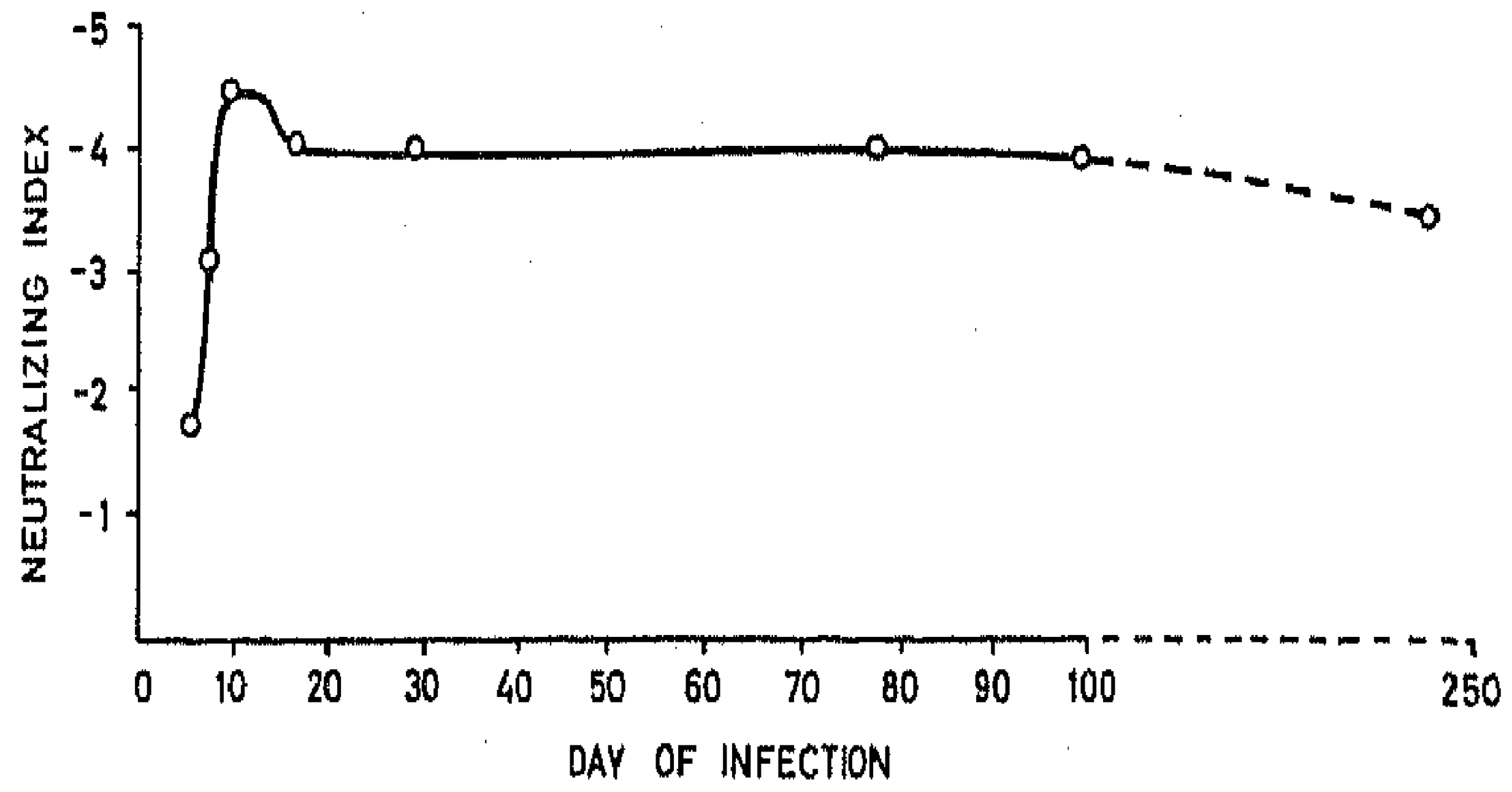

FIG. 2. Neutralizing antibody titres in sera from bullock 840, using EATRO-3 as antigen.

TABLE V. NEUTRALIZATION OF TRYPANOSOME STABILATES FROM BULLOCK No. 840, BY SERA FROM THE SAME ANIMAL

STABILATES	Serum						
	Day [x]	0	10	32	53	74	226
	14	0	0	+	+	+	+
	35	0	0	0	+	+	+
	56	0	0	0	0	+	+

+ Positive agglutination or neutralization.
0 Negative agglutination or neutralization.
x Figures refer to the day of infection on which serum was collected or trypanosomes were isolated.

(c) Results obtained using the agar-gel double diffusion test

Using EATRO-3 as antigen, lines of precipitation were detected as early as the 10th day after infection and increased in number and intensity as the disease progressed, until at least the 150th day. When serial stabilates from an animal were compared with each other, and with the infecting stabilate (EATRO-3), identical lines of precipitation were observed. These results imply the presence of common antigens in EATRO-3 and the isolates obtained at intervals during infection in the cattle. Figure 3 shows results obtained by testing serial serum samples from the cattle against EATRO-3 antigen.

(d) Results obtained using the indirect fluorescent antibody test

This test was applied only to material collected from Bullock No. 480. Using EATRO-3 as antigen, antibodies were detectable during the second week of infection and remained present until at least the 320th day after infection.

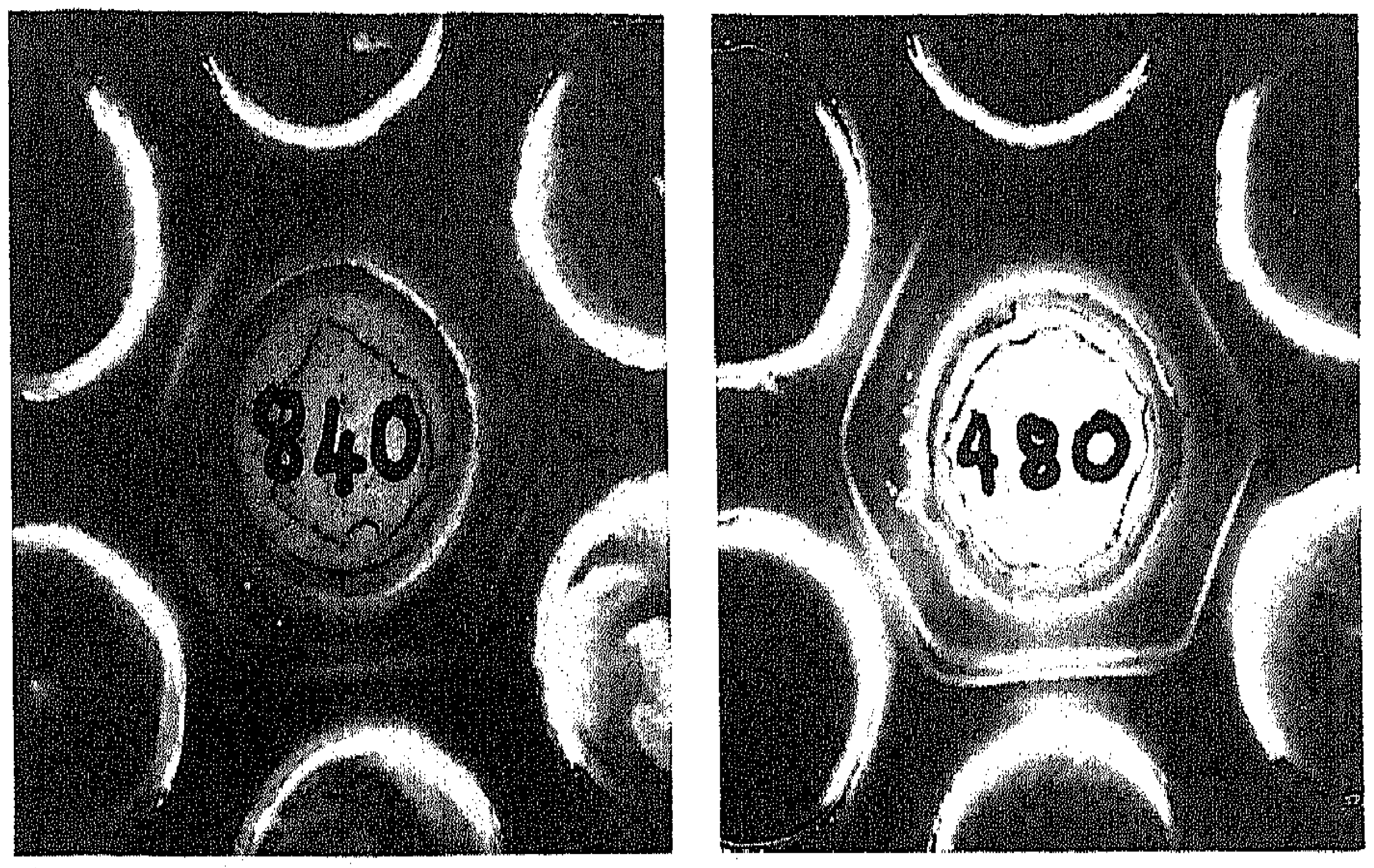

FIG. 3. Agar gel double diffusion, with antigen prepared from EATRO-3 in the centre well. From 12 o'clock, moving clockwise, serum before infection and serum 30, 60, 90, 120 and 150 days after infection, respectively.

Using five of the serial isolates as antigen, all gave positive results with sera which were obtained between the 27th and 140th days after infection (see Table VI).

Results obtained from investigations concerning Bullock No. 5, infected with EATRO-691

A relapsing parasitaemia was observed, with peaks at the 7th day and the 13th day. It should be noted that too few trypanosomes were present to use visual counting techniques to detect the changing parasitaemia; only the greater sensitivity of the infectivity titration technique made this observation possible. The results are shown in Fig. 4.

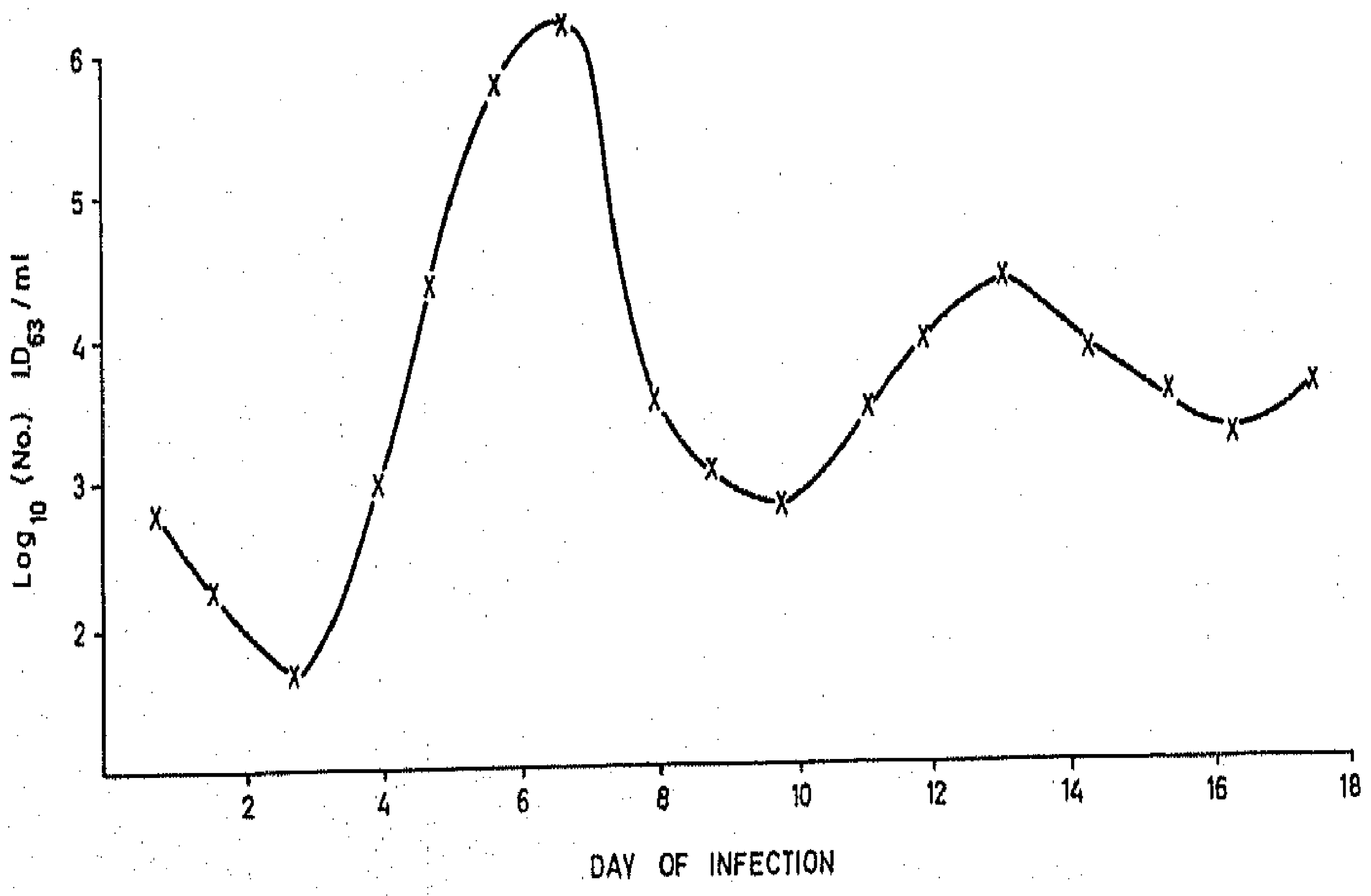

FIG. 4. Parasitaemia in bullock 5, results obtained by titration of fresh blood samples in mice.

TABLE VI. TITRES OBTAINED USING FLUORESCENT ANTIBODY TECHNIQUE, WITH SERUM AND TRYPANOSOME STABILATES OBTAINED FROM BULLOCK No. 480

	Day x	Trypanosome stabilates				
		7	28	56	84	140
S	6	-ve	-ve	-ve	-ve	-ve
E	27	1/320	1/160	1/80	1/320	1/160
R	63	1/320	1/320	1/160	1/320	1/160
U	84	1/320	1/320	1/320	1/160	1/160
M	140	1/160	1/160	1/160	1/160	1/160

x Figures refer to day of infection on which serum was obtained or trypanosomes were isolated.

TABLE VII. NEUTRALIZATION OF STABILATES FROM BULLOCK No. 5, BY SERA FROM THE SAME ANIMAL. NUMBERS SHOW NUMBER OF MICE/6 BECOMING INFECTED

Antigen	Daily serum samples																	
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
EATRO-691	6	6	6	6	3	5	2	1	0	0	0	0	0	0	0	0	0	0
7-day stabilate	6	6	6	6	6	6	6	6	6	3	1	0	0	0	0	0	0	0
13-day stabilate	6	6	6	6	6	6	6	6	6	6	6	6	6	6	4	2	0	0

The results of the neutralization tests using EATRO-691, the 7th day stabilate, and the 13th day stabilate, against the daily serum samples are shown in Table VII.

Results obtained from investigations concerning Bullocks Nos 23, 167, 985 and 59

Agglutinin production to the homologous stabilate in each of the Bullocks Nos 23, 167 and 985 was similar to that observed in cattle Nos 840, 480, 612 and 616 against EATRO-3 (see Fig. 1), except that agglutinins were first detected on the fourth day, and reached a peak by the tenth day.

In cross agglutination tests, positive results were obtained with homologous systems only, when serum dilutions greater than 1/20 were used. Agglutination occurred with stabilates 480/1 and 480/9 using serum from Bullock No. 167 at dilutions up to 1/20.

Using the qualitative neutralization test, positive results were obtained with homologous systems only.

The results of the agar-gel double diffusion tests are shown in Fig. 3, demonstrating the presence of common antigens in stabilates 480/1, 480/5 and 480/9, and the production of antibodies against them by the cattle infected with EATRO-3.

High titre agglutinins and neutralizing antibodies were produced simultaneously by Bullock No. 59 against each of the inoculated stabilates, implying that each of the antigenic variant populations may comprise more than one antigenic type.

DISCUSSION

During infection in cattle, it would appear that T. brucei subgroup organisms produce a succession of antigenic variant populations probably at less than seven-day intervals, each of which are produced only once during the infection, that this process continues for at least 20 weeks, and probably for the duration of the infection. In addition, it has been demonstrated that these antigenic variant populations have antigens in common. These conclusions, that T. brucei subgroup organisms produce variable and common antigens, are supported by the exhaustive investigations of Williamson and Brown [12]. It should be noted that Anti-32, one of the stabilates used by Williamson and Brown [12], was also a derivative of EATRO-344.

In response to the antigenic variant populations produced by the trypanosomes, cattle produce a succession of homologous agglutinins and neutralizing antibodies, while antibodies against the common antigens are produced within two weeks of infection and are thereafter continuously present throughout the infection.

It will be noted that some of the results do not agree with the above conclusions. For example, in the case of Bullock No. 480, the 32-day serum samples agglutinated trypanosomes isolated from the same animal much later in the infection, implying that at least some of the antigenic variant populations may reappear. It is considered, however, that the method used in the present investigation for the isolation of antigenic variants from the cattle, namely inoculation into mice, is not sufficiently critical to ensure the stability of the antigenicity of the organisms. It is quite possible that antigenic variation may take place while the organisms are being passaged in mice. The results obtained using the neutralization test with samples obtained from Bullocks Nos 5 and 850 indicate that neutralizing antibodies against each antigenic variant population are quickly produced, and remain detectable for extended periods. The continued presence of specific neutralizing antibodies against particular antigenic variants would appear to preclude their reappearance.

In mild trypanosome infections it is probable that there is a delicate balance between the host and the parasite, and as long as the host is able to produce neutralizing antibodies against the antigenic variants produced by the parasite, the balance will be maintained and the parasites will eventually die out. Any factor which interferes with the ability of the host to produce antibodies could be expected to destroy this balance. In support of this hypothesis it has been widely reported that animals in apparent good health, but with a demonstrable trypanosome parasitaemia, will succumb if stressed. For example, when a virulent rinderpest vaccine is used in a trypanosomiasis enzootic area, many cattle die, apparently of trypanosomiasis,Hornby, Evans and Cornell [13]. It has also been reported, Lumsden [14], that wild animals may die soon after capture, with a terminal trypanosome parasitaemia.

It is considered probable that in cattle infected with *T. brucei* group organisms the release of common antigens from the successive antigenic variant populations and their reaction with homologous antibodies may be of importance in the pathogenesis of the disease.

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VACCINATION OF CATTLE AGAINST TRYPANOSOMES BY INFECTION AND TREATMENT

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Abstract

VACCINATION OF CATTLE AGAINST TRYPANOSOMES BY INFECTION AND TREATMENT. Six animals were infected with *Trypanosoma brucei*, type EATRO-2/1, and six kept as controls. Infected animals were parasitaemic seven days later. Fourteen days after inoculations all animals were given Berenil, 7 mg/kg body weight: two cattle in each group were then challenged with the inoculation dose of EATRO-2/1, two or three months after Berenil treatment. Vaccinated animals showed a much higher degree of resistance, only one being parasitaemic on one occasion. Among the controls all but one became parasitaemic. Animals challenged at one month were again challenged eight months after Berenil treatment, resulting in parasitaemia in controls while the vaccinated animals were negative. Neutralizing antibodies were demonstrated in vaccinated animals up to eight months after Berenil treatment but on no occasion in controls.

Many authors have reported that when laboratory rodents are cured of trypanosomiasis by drug treatment they resist challenge with the same strain of organisms for considerable periods. It has also been observed that cattle cured of the natural infection survive in enzootic areas without further treatment, this phenomenon being attributed to an immune response. Attempts to produce an immune response in cattle by the repeated exposure of animals to infection by trypanosomes, followed by treatment, have not been successful, mainly because due care was not taken to control either prophylactic activity of the drug used for treatment nor antigenic variation of the organisms.

In this communication an experiment is described in which cattle were infected with trypanosomes of the *T. brucei* subgroup, and treated 14 days later with Berenil. Neutralizing antibodies against the organisms used to infect the cattle were demonstrated in the serum of the cattle, and also the cattle resisted challenge for up to eight months, while control animals became parasitaemic within seven days.

MATERIAL AND METHODS

Trypanosomes

Trypanosoma brucei group organisms (EATRO-2/1) were used. EATRO-2/1 was isolated in mice from naturally infected *Glossina pallidipes*, caught at Lugala, South Bukedi, Uganda, and after seven passages in mice was preserved at approximately -80°C, as described by Cunningham et al. [1].

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Cattle

Adult East African Shorthorn Zebus from the EATRO farm were used. Prior to the experiment they were shown to be free of trypanosomiasis by slide examination and inoculation of blood into mice.

Mice

Swiss white mice from the EATRO Colony were used.

Measurement of infectivity of trypanosome suspensions

The technique described by Lumsden et al.[2] was used.

Six cattle were inoculated intravenously with 10 ml of a 10^{-2} suspension of stabilate EATRO-2/1. By titration in mice it was established that this dose contained approximately 10^6 ID_{63}. All the cattle were shown to be parasitaemic within seven days, by the inoculation of blood into mice, which subsequently became infected. Fourteen days after the inoculation of EATRO-2/1 all the cattle were treated with Berenil at a dose-rate of 7 mg/kg body weight. At the same time another group of six uninfected cattle were treated with Berenil at the same dose-rate.

Two cattle in each group were challenged one, two, and three months after Berenil treatment with the same dose of EATRO-2/1 (proved by titration in mice on each occasion) as was initially used to infect the cattle. To establish the presence or absence of infection blood from each of the cattle was inoculated into mice at weekly intervals for the duration of the experiment. Serum was collected from the cattle at regular intervals for use in the neutralization test. To establish the presence or absence of infection blood from each of the cattle was inoculated into mice at weekly intervals for the duration of the experiment.

RESULTS

Of the cattle challenged one month after treatment one of the control animals was parasitaemic on one occasion and subsequently remained negative. None of the remaining three cattle became infected.

Of the cattle challenged two months after treatment both controls became parasitaemic and also one of the vaccinated animals. The other vaccinated animal did not become infected.

Of the cattle challenged three months after treatment both controls became infected while both vaccinated animals remained negative.

The four animals which were challenged one month after treatment, and failed to become infected, were challenged again eight months later. Both the control animals were parasitaemic within seven days while both vaccinated animals remained free of infection.

Using EATRO-2/1 as antigen, neutralizing antibodies were demonstrated in the serum of the vaccinated animals for up to eight months after treatment, while antibodies were never detected in the control animals, either before or after challenge.

DISCUSSION

It has been shown earlier that Berenil at trypanocidal levels can be demonstrated in the serum of cattle three weeks after treatment at a dose-

rate of 7 mg/kg body weight, and it is probable, from the results of this experiment, that protection against fairly light challenge lasts for at least one month, but less than two. Since all the control cattle which were challenged two or more months after treatment became infected, it is concluded that the vaccinated animals developed an acquired immunity against EATRO-2/1.

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IMMUNOLOGICAL STUDIES ON *FASCIOLA HEPATICA*

Clinical and Immunological Response in Mice to Infection with Irradiated and Normal Fluke Cysts

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Abstract

IMMUNOLOGICAL STUDIES ON *FASCIOLA HEPATICA*: Clinical and immunological response in mice to infection with irradiated and normal fluke cysts. In this paper the relevant literature is reviewed critically. Preliminary results on the clinical picture, haemagglutinating and complement-fixing antibody response as well as to the effect on miracidia of sera of mice infected with normal and irradiated metacercariae of *F. hepatica* are reported. Future lines of immunological studies on liver fluke are also discussed.

The results obtained are as follows: (a) the clinical picture was characterized by a marked loss in body weight, in the range of 23 to 30%, two weeks after infection in mice dosed with five normal, or with 20 or 100 irradiated cysts. An almost complete regain in body weight was found six weeks following the infection, except in mice dosed with 5 normal cysts. These still had a loss of 22%. The number of red cells decreased from an average of 7.7 millions/ml to 4.0, 4.1 and 3.7 millions/ml, respectively, three to four weeks after the above-mentioned infections. However, variations in the number of white cells, from 3400 to 19 900/ml, compared with 9300/ml in controls, were observed. Lymphocytosis, which was established, reached its peak three weeks after the second dose of 20 or 100 irradiated cysts. Basophil leukocytes were not noted; (b) serologically, no haemagglutinating antibodies were found in any group of experimental mice. Complement-fixing antibodies, when stimulated by using infection with either normal or irradiated cysts, disappeared from the blood four weeks after infection, with only one exception related to the 100-cyst dose. A repeated infection of the groups of mice, four weeks later, with 20, 50 and 100 irradiated cysts, resulted in increased titres of complement-fixing antibodies. The titre of 160, expressed as a reciprocal of the dilution, was established, irrespective of the above doses, four weeks following the second infection. Longer time intervals between infection and other variables have not been explored as yet; (c) the significance of the fluke infections in mice, by using the miracidial in-vitro test, is in progress and the result of this investigation will be presented later.

INTRODUCTION

Several workers have studied the immunological response to *Fasciola hepatica* in animals. Most of the research work done in the last two or three decades has been reviewed by Dawes and Hughes [1], and Kagan [2]; Soulsby [3] also reviewed a part of the relevant literature, which will be critically summarized as follows:

Natural and acquired resistance to liver fluke was explored by Montgomerie [4], who found that experimental infection persisted in rabbits. Leiper [5] and Taylor [6] found no evidence of post-infection resistance in goats or sheep, respectively. Durbin [7] established that adult flukes survived in the bile ducts of experimentally infected sheep for more than 11 years. Recently Sinclair [8] reported that several doses of infection did not prevent the successful migration of F. hepatica in sheep liver "but there was evidence of delayed onset and reduced egg production by later-maturing adults". Montgomerie pointed out that cattle may possibly react differently from sheep to infection with liver fluke. He stated that, in spite of severe infection, livers of cattle $2\frac{1}{2}$ years old generally showed no evidence of "the presence of a single fluke". Dawes and Hughes [1], referring to Lederman's [9] work reported that the author believed that the stock in an infected area in the Democratic Republic of the Congo had attained appreciable tolerance to F. hepatica and would free themselves from the flukes in one or two years after having been removed from the source of infection.

Some serological and immunochemical work has also been done on F. hepatica. Hughes [10], using an immune diffusion technique in agagel, was mainly concerned, in a part of his serological work, in finding antigens that might produce resistance to F. hepatica infection in experimental animals. Cysts and non-living antigens of fluke origin, the latter also together with Freund's complete adjuvant, were used in the immunization of rabbits. Although a marked antibody response was established in the vaccinated rabbits by the above technique, these animals failed to show any significant resistance to oral challenge with cysts. Sewell [11, 12] studied autofixation of a guinea-pig complement and the qualitative precipitin reaction in fascioliasis. Biguet et al. [13, 14] demonstrated a mosaic structure of F. hepatica soluble antigens consisting of at least 15 fractions, some of which are common to the other helminths.

Passive immunization and the effect of "immune" sera were also explored. Soulsby [15] demonstrated antagonistic and lethal effects on miracidia of sera of sheep infected with mature and immature liver flukes as well as those of non-infected sheep. Wikerhauser [16] observed that microscopically visible precipitates formed at the natural orifices of the young artificially excysted flukes immersed in rabbit or bovine "immune" guinea-pigs Wikerhauser [16], as well as intraperitoneal injection of mice with sera of rabbits previously infected or vaccinated with F. hepatica, Hughes, [10] failed to influence the course of oral infection of the host with the fluke cysts. Wikerhauser [16] found, however, that young fluke forms excysted in vitro caused a milder infection when placed in the guinea-pig abdominal cavity after immersion in "immune" serum or when the serum was injected intraperitoneally at the time of an intraperitoneal infection.

Vaccination against F. hepatica was first carried out by using non-living fluke materials, Kerr and Petkovich [17] vaccinated rabbits intraperitoneally with dried adults of F. hepatica. Comparing the mean fluke number in vaccinates with that in controls, resulting from a challenge infection, these workers established the ratio 2 : 8 in favour of the vaccinates, thus indicating that it was possible to attain a degree of immunity. Urquhart et al. [18] injected rabbits with alum-precipitated purified protein antigen. They found smaller fluke forms from injected rabbits in comparison to controls, but there was no significant reduction in fluke numbers. Healy's [19] immunological work in rabbits injected with F. hepatica tissue extracts or homogenized caecal contents yielded no evidence of resistance to infection. According to the review by Dawes and Hughes [1], Shibanai et al. [20] injected rabbits with an F. hepatica antigen by various routes previous to oral infection with fluke cysts. This work resulted in the finding of macroscopically and histologically less severe liver lesions, in comparison to controls, in animals injected subcutaneously and intradermally. Ershov [21] presented evidence of immunity to infection with F. hepatica in 10 to 25% of sheep which were immunized by a polysaccharide complex. Hughes [10, 22] obtained a statistically significant degree of protection to F. hepatica infection in rabbits immunized with lyophilized adult fluke antigens. Post-vaccinal oral infection yielded a mean fluke number of 1.7 per vaccinated rabbit as against 5.6 per control. Repeat experiments failed, however, to demonstrate any degree of immunity. The author also vaccinated sheep but obtained statistically significant differences neither in the time of patency nor in the mean numbers of fluke resulting from the post-vaccinal infection compared to controls. The ratio of the two groups was 59.6 : 78.2.

More recently, living normal and irradiated metacercariae have been used to study the immune response to F. hepatica infection. However, little immunological work has been published as yet on irradiated liver fluke forms. Thorpe and Broome [23] vaccinated rats each with 40 metacercariae irradiated with various doses of X-rays within the range 1 to 10 kR. Rats were challenged orally with 20 cysts each 7 or 11 weeks later and necropsied 10 weeks following challenge infection. On the basis of the established worm burden the authors claimed that there was evidence of attained immunity in rats dosed with 40 metacercariae irradiated at 1 kR, and "significant immunity" at 2.5 kR doses "with a reduction of approximately 50% in challenge take at both 7 and 11 weeks after immunization". Comparison of this experimental group with worm numbers of a control group from a previous experiment, however, makes the reported results questionable. Hughes [10] dosed mice doubly at a three-week interval; each dose contained 20 cysts irradiated at 4 kR of X-rays. Three weeks after the second dose these mice, together with untreated controls, were challenged orally, each with 10 normal cysts. At the same time mice treated only with irradiated cysts served as "attenuation" controls. This experiment, however, provided no evidence of immunity. Mice, in a subsequent experiment, were dosed doubly at the same interval as before but each dose consisted of 40 metacercariae irradiated at 3 kR. Experimental animals and controls were challenged seven weeks after the second dose. A mean worm burden of 3.5 flukes per experimental mouse as against 4.0 flukes per control were obtained. Dawes (see: Dawes and Hughes [1]) repeated, in part, the foregoing experiments with 40 cysts irradiated at 3 kR of X-rays and reported: "In the results of neither series of experiments in mice, therefore, was there any evidence of immunity in terms of intensity of infection" and he added "there was no immunity in terms of delayed maturity either". Hughes [10] failed also to immunize rabbits with two doses of 500 metacercariae irradiated at 4 kR. The interval between doses was three weeks, and four weeks between a second dose and challenge with 15 normal cysts. Applying essentially the same vaccination schedule, Hughes attempted to immunize sheep with irradiated cysts. These were challenged, each with 200 metacercariae seven weeks following the second vaccine dose. The results obtained furnished no evidence of significant difference between the two groups of sheep in terms of recovered worm burdens, these being 66.6 flukes per vaccinate as against 79.2 per control.

Most recently Lang [24] immunized experimental mice by two infections each with two normal metacercariae of F. hepatica. The second immunizing infection was separated from the first by 60 days. Challenge infection of the same size was administered to the experimentals and untreated controls 40 days after the second infection. The mice were observed and examined at scheduled intervals for 40 days following challenge. On the basis of various data, which were obtained, primarily on histopathology of the livers where timing and nature of lymphocyte infiltration were investigated, a significantly smaller fluke number was recovered from the experimentals as compared with controls. In the experimental mice flukes reached the common bile duct at least 10 days earlier than in the control ones. The author suggested "that delayed (cellular) hypersensitivity may play a prominent role in the earlier migration of worms to the common bile duct". He assumed this to be the result of acquired immunity.

From the critical summary of a larger number of the relevant papers it can be inferred that at present it is not possible to formulate any definite conclusion on the immune status to fascioliasis. It appears that at present there is not available, in immunological studies, sufficient information on some possibly important factors, such as relative importance of irradiated flukes as the source of antigens, dose and time of its administration, host species responsiveness to the liver fluke infection, pathogenesis and histopathology of several subsequent infections, and also of the role of the liver and Kupffer cells in these infections. The effect of sera of infected host species on immature flukes has not been extensively explored. Our present knowledge of techniques which may be used in these immunological studies seems to be limited, too. The aim of this paper is to present some preliminary results obtained in mice which may be of interest in further work and also to discuss lines of immunological research in infections with F. hepatica.

MATERIAL AND METHODS

Miracidia and metacercariae of F. hepatica and albino mice represented the living biological material in these experiments. Sera, antigen, complement and haemolysin comprised other biological ingredients. This material has been described earlier (Sokolić [25]).

Techniques used in serological assessing of the results were: (a) passive haemagglutination as described by Boyden [26]; and (b) complement-fixation based on the principles given by Mayer [27] and Kent and Fife [28]. Titres of complement-fixing antibodies were calculated on the basis of 50% endpoint haemolysis as measured at 530 m in a "Unicam SP 500" spectrophotometer, using 1-ml cells. The biological miracidial test was carried out as described previously, Sokolić [25]. Cysts were irradiated with X-rays under conditions reported by Movsesijan [29].

CLINICAL EXAMINATION

The mice were examined clinically before infection and at one-week intervals thereafter. They were weighed and their quantitative and qualitative blood picture was determined during an experimental period of eight weeks.

RESULTS

I. Clinical picture in mice

Body weight, quantitative and qualitative blood picture, haemoglobin concentration and death rate were examined comparatively in three groups of mice, each comprising 120 animals at the beginning of the experiment. The mice of the above experimental groups were infected each with 5 normal or with 20 or 100 cysts irradiated at 3 kR. Four weeks later one half of the mice in each group were reinfected with the same numbers of cysts. Thirty non-treated animals served as controls. The results, representing means obtained from 4 or 5 mice in each of six subgroups, are described briefly. The mice were examined weekly over eight weeks following the infection.

(a) Body weight in mice decreased markedly two weeks after infection, when the loss in the aforementioned three groups of mice was respectively 28, 23 and 30% of the body weight in non-treated controls. Thereafter, body weight increased slowly and reached starting values of 24 to 28 g six weeks after infection, except in mice dosed with five normal cysts. These had still lost 22% of their body weight at the beginning of the experiment.

(b) The number of red cells decreased from 7.7 millions/ml in controls to 4.0, 4.1 and 3.7 millions/ml in mice infected with 5 normal, with 20, and 100 irradiated cysts, respectively. The figures represent the lowest numbers of red cells established in this experiment and were obtained at the end of the third post-infection week for the mice dosed with 100 irradiated cysts, and at the end of the fourth week for the remaining two subgroups of animals. The numbers of erythrocytes varied similarly in the subgroups of reinfected mice.

(c) The number of white cells varied greatly compared with controls, which had, on average, 9300 white cells/ml. The variations ranged from about 3400/ml found in mice four weeks after infection with five normal cysts to 19 900/ml in mice four weeks following reinfection with 100 irradiated cysts. The qualitative blood picture was characterized by lymphocytosis observed in mice after the second dose of 20 or 100 irradiated cysts. It reached a peak in the seventh experimental week. The number of eosinophils varied from 0 to 11%. Exceptionally, it approximated 19% in the group of mice dosed with 100 irradiated cysts. No basophilic leukocytes were observed in any of the mice examined.

II. Immune response

(a) Serological testing

The formation of haemagglutinating and complement-fixing antibodies was explored after the infection of mice. Two experiments, comprising 486 mice, were undertaken. In a preliminary experiment 56 mice were allotted in groups, as shown in Table I. No haemagglutinating antibodies

TABLE I. TITRES OF COMPLEMENT-FIXING ANTIBODIES IN MICE INFECTED WITH NORMAL OR IRRADIATED FLUKE CYSTS

Mice (No.)	Cysts given (No.)		Complement fixation test						
	Normal	Irradiated	Time of sample (weeks post infection)	Titre 10	20	40	80	160	320
4	5	-	2	++	+	-	-	-	-
4	10	-	2	+	+	-	-	-	-
4	-	50	2	++	++	+	+	+	-
4	-	-	2	-	-	-	-	-	-
4	5	-	4	+	-	-	-	-	-
4	10	-	4	++	+	+	+	-	-
4	-	50	4	++	++	+	+	-	-
4	-	-	4	-	-	-	-	-	-
4	-	50	6	-	-	-	-	-	-
4	-	50 + 50 a	6	++	++	++	+	-	-
4	-	-	6	-	-	-	-	-	-
4	-	50	8	-	-	-	-	-	-
4	-	50 + 50 a	8	++	++	++	+	+	-
4	-	-	8	-	-	-	-	-	-

[a] The interval between infections was four weeks.

TABLE II. TITRES OF COMPLEMENT-FIXING ANTIBODIES IN MICE INFECTED WITH NORMAL OR IRRADIATED FLUKE CYSTS

Mice (No.)	Cysts given (No.)		Complement fixation test						
	Normal	Irradiated	Time of sample (weeks post infection)	Titre 10	20	40	80	160	320
4	5	-	1	-	-	-	-	-	-
4	-	20	1	-	-	-	-	-	-
4	-	100	1	-	-	-	-	-	-
4	-	-	1	-	-	-	-	-	-
4	5	-	2	-	-	-	-	-	-
4	-	20	2	+	-	-	-	-	-
4	-	100	2	+	-	-	-	-	-
4	5	-	3	-	-	-	-	-	-
4	-	20	3	+	+	+	-	-	-
4	-	100	3	+	+	+	-	-	-
4	5	-	4	+	-	-	-	-	-
4	-	20	4	+	+	-	-	-	-
4	-	100	4	+	+	+	-	-	-
4	-	-	5	-	-	-	-	-	-
4	-	20 + 20[a]	5	++	-	-	-	-	-
4	-	100 + 100[a]	5	++	+	-	-	-	-
4	-	20 + 20[a]	6	++	+	-	-	-	-
4	-	100 + 100[a]	6	++	++	++	+	-	-
4	-	20 + 20[a]	7	++	+	+	+	-	-
4	-	100 + 100[a]	7	+++	++	++	+	+	-
4	-	-	8	-	-	-	-	-	-
4	-	20 + 20[a]	8	++	++	+	+	+	-
4	-	100 + 100[a]	8	+++	++	++	+	+	-

[a] Interval between infections was four weeks.

were found in these experimental mice. The result relating to complement-fixing antibodies is shown in Table I.

A larger-scale experiment, comprising 390 mice allotted in three groups of 120 each and 30 controls, was next carried out. The experimental mice were infected as described under 'I Clinical picture in mice' each one with 5 normal, or 20 or 100 irradiated cysts. Half the number of mice in the latter two groups were reinfected with 20 or 100 irradiated cysts four weeks later (see Table II). Four or five mice from each infected group and three or four controls were bled weekly over eight weeks. Pooled sera were titrated as previously. Negative titres were obtained in respect of haemagglutinating antibodies. Most of the results of the complement-fixing test are presented in Table II.

Summing up the results of serological tests the following can be concluded: (i) no haemagglutinating antibodies were found over eight post-infection weeks in the sera of mice dosed either with normal or irradiated fluke cysts; (ii) it has been established that both infections, with normal and irradiated cysts, stimulated the production of complement-fixing antibodies. Antibodies resulting from a single cyst dose disappeared from circulation after the fourth post-infection week except those of mice infected with 100 irradiated cysts, which resulted four weeks later in an antibody titre of 160, irrespective of the above cyst doses. The negative titres for weeks 5 to 8, resulting from single infections, are not presented in Table II, nor is the titre 10 from 100 irradiated cysts.

(b) Biological in vitro testing

It is generally assumed that juvenile worms are more susceptible than adults to the possible adverse effect of sera of animals with homologous infections. To investigate biologically the significance of the infections in mice, described above, freshly hatched miracidia were immersed in the sera of mice. Although the work is still in progress, preliminary results have indicated that the miracidial in-vitro test appears to be a suitable procedure to examine the antagonistic effects of sera of infected mice as against untreated controls. The intensity of serum antagonistic reactions seems at present to be correlated with the complement-fixing antibody titres.

DISCUSSION

Antigens of liver fluke are highly important in the studies on acquired immunity to this trematode. Soulsby [3] expressed the view that, under normal conditions, each fluke, during its migration in the liver, contributes only small quantities of protective antigens. Quantities sufficient to stimulate protective immunity would be provided perhaps only in a lethal dose.

If the above view is correct, further immunological studies could be possible when sufficient quantities of protective antigens are available from the in vitro culture of immature flukes. Alternatively, irradiated fluke forms, now available, could be used in these studies, although a knowledge exists of the differences in the structure and biology of normal and irradiated cysts. Reduced pathogenicity in irradiated forms has

been established and mice withstood a repeated infection with 100 irradiated cysts in contrast to as low infection as five normal cysts, LD_{50} for the latter being a mean interval of 26 days, Sokolić [25]. From this it would appear that irradiated cysts may induce initially a larger antigenic stimulation in the host owing to its withstanding a significantly larger dose of infection. Comparative studies of normal and irradiated cyst infections, Sofrenović et al. [30], have shown marked qualitative differences in the histopathology of these infections. Histopathology of the former infection was generally characterized by progressive destruction of the liver parenchyma and the absence of reactive cells; the latter infection, on the contrary, resulted in demarcation and encapsulation of destroyed foci, in proliferation of mesenchymal cells and the appearance of numerous active immunocysts. Disintegration of irradiated flukes gives an indication of possibly altered antigenic stimulation in which particules of irradiated cysts, or active fragments, may also play a role. A question which arises in the further speculation on irradiated fluke antigen is: whether a sublethal dose of radiation in metazoan organisms stimulates, similarly to mammalian cells, restorative processes by specific protein synthesis from rich pools of nucleic acid derivatives liberated from the radiation-damaged cells during their catabolism degradation (see: Taliaferro et al. [31]). Specific protein synthesis in sublethally irradiated helminths might, perhaps, also add to the explanation for the stimulation of protective immunity, at least by some of the helminths.

From the above it appears that irradiated cyst infections and the mechanism involved in inducing immune response may differ from infections with normal cysts. This, in addition to the studies of the fluke antigen complex structure, its fractionation, isolation, and identification of antigenic components, might be a part of a long-term research programme closely connected with obtaining more information and knowledge on bio- and immunochemistry, radiation and immunobiology in general, and, in particular, on this trematode. However, continuation of the investigations on the immunizing schedule to F. hepatica may be our immediate research programme. This can include a number of variables, such as normal and irradiated material, its dose, time intervals and routes of antigen administration, in addition to the studies of liver physiopathology and immunopathology, restorative and immunocytic processes associated with fluke infections. Further exploration of the Kupffer cells on a subcellular level should add to a better understanding of their role in this complex immunological response of the host. Both humoral and cellular host components should be investigated adequately. Such observed phenomena as juvenile fluke immobilization, precipitation, agglutination, disintegration in contact with the sera of the infected host, cellular attraction, or adhesion to early fluke stages, delayed hypersensitivity and a number of other reactions, may be indicators of a possible protective immune response to F. hepatica infection. Fractionation of certain host humoral and cellular components and the concentration and transfer of the most active ones of these to recipients so as to investigate the possibility of passive immunization would also be a way for presenting evidence of protective antibodies. Diffusion chambers, fluorescent and radioisotope labelling, and a number of other techniques, may be of great value in these studies.

In conclusion, the above suggestions are only a part of what, perhaps, has to be accomplished. The enthusiasm of each research worker, common co-operation and the best co-ordination in the work, are needed to speed elucidation of the immunology of fascioliasis.

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EFFECTS OF TOTAL- (PARTIAL-) BODY IRRADIATION ON HELMINTHIC INFECTIONS

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Abstract

EFFECTS OF TOTAL- (PARTIAL-) BODY IRRADIATION ON HELMINTHIC INFECTIONS. The effects of irradiation of the body on sensitivity and resistance to viruses and schizomycetes have been studied on numerous occasions following the discoveries of L. Hectoen. This is a complex problem of which various aspects still remain to be explained, for example the effects of such important factors as the nature of the radiation and the form in which it is administered, the lapse of time between injection of the antigen and irradiation, and so on.

In the sphere of microbiology numerous authors have conducted research on the effects of irradiation of the body on natural or acquired immunity. On the other hand, the effects of irradiation of the body on animals suffering from helminthic parasitization have not been extensively investigated.

Interesting results have been obtained from studies carried out on animals previously immunized, irradiated and subjected to post-irradiation tests, such as titration of the antibodies or control contamination. These studies reveal partial or total loss of acquired immunity following exposure to high doses.

The investigations carried out by the author of the paper – the first part of which is completed – relate to whole-body exposure to X-rays ($LD_{50/30}$) of guinea pigs parasitized after irradiation with 1500 embryonnated eggs of ascaris suum. The results – which have been set forth in greater detail elsewhere – are in agreement with those obtained by other workers. The mortality among the guinea pigs first irradiated and then parasitized proved to be very high during the first ten days, autopsy showing serious pulmonary haemorraghias. In the case of guinea pigs irradiated but not parasitized the mortality was lower (50% in 30 days); zero mortality was observed in guinea pigs parasitized but not irradiated. Among the irradiated animals, the migration of larvae in the liver and lungs was at least as rapid as in the control animals which had been parasitized only; the number and size of the larvae were the same as in the control animals.

The effects of body irradiation on receptivity and resistance to viruses and schizomycetes were studied by numerous authors after Hektoen's findings [1] (see Taliaferro and Taliaferro [2]). It is a complex problem in which many questions remain to be answered, Trum and Rust [3], especially in connection with a number of variables, among which entity and form of ray administration and time relationship between antigen injection and irradiation are very important.

In the microbiological field, in particular, much research has been done on the effects of irradiation on innate (natural) and acquired immunity[1].

As regards innate immunity, this is suppressed, Taliaferro, Taliaferro and Jaroslow [4], by high doses of radiation. Such suppression is better documented in those infections against which the host presents high natural immunity. The depression of natural unsusceptibility of the host to microbial infections is already patent in the latent period (i.e. 1 to 2 days after irradiation), Klemparskaya et al. [5]; higher receptivity to microbial infection is present, nevertheless, up to 15 days after irradiation, Hammond [6], Klemparskaya et al. [5], Schechmeister et al. [7].

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[1] The effects of radiation on passive immunity are not examined here because it has not been investigated in the helminthological field until now.

In acquired immunity there are two immune responses, the primary response in hosts not previously injected with antigen and the secondary response in hosts previously sensitized.

The immune primary response (see Taliaferro et al. [4]) is positive when antigen is given at critical times before the administration of large cytotoxic doses of X-rays, Dixon and McConahey [8]. Such positive responses were reported by Dixon and McConahey [8] in rabbits injected with antigen $2\frac{1}{2}$ days before exposure to 600 R, and in rabbits injected with antigen 2 hours before exposure to 450 R by Taliaferro and Taliaferro [9], by Taliaferro, Taliaferro and Janssen [10] in animals injected with antigen 1 to 4 hours before large doses from X-rays; and by Gengozian and Makinodan [11] in animals injected with antigen five days before exposure to 710 R; etc.

Enhanced antibody titres were also reported when large doses of X-rays were administered to a local site not long after the antigen injection, Graham et al. [12], Graham et al. [13]. Small doses of radiation locally administered were equally positive, as was shown by Schaefer [14] on the course of subcutaneous staphylococcic infection in the rabbit; by Fried [15] on the course of pneumonia experimentally induced in the guinea-pig; by Kelly and Dowell [16], Dowdy and Sewell [17], and Cantril and Buschke [18] on the course of gas-bacillus infections.

On the contrary, many other authors, Gengozian and Makinodan [19], Kohn [20], Salerno et al. [21], Stoner and Hale [22] did not find enhanced antibody titres, but normal titres and subnormal antibody responses, when antigens were injected several hours to several days prior to irradiation.

However, in this respect, various factors are very important (see Taliaferro et al. [4]), for example, methods of antigen injection; the kind of antigen (living, i.e. able to multiply, or non living); methods of administration of rays (single or multiple doses), etc.

The primary antibody response is inhibited when antigen is injected not long after X-irradiation, and the loss of antibody-forming capacity is maximal when antigen is injected one to two days after irradiation, Taliaferro et al. [4].

The secondary antibody response (in animals sensitized with antigen from one to four months before) was studied by Gengozian and Makinodan [19] and by Makinodan et al. [23]. The antibody-forming capacity is normal but not high when antigen is injected before a sublethal dose of X-rays; the depression of this capacity is maximal when antigen is injected one to two days after X-irradiation and such depression increases as the dose of X-rays is increased.

Many explanations are given on antibody-forming capacity - especially the loss of this capacity - of organisms subjected to stress by ionizing radiation; this is chiefly because little is known about radiosensitivity on the various steps of antibody formation, Klemparskaya et al. [5]. Certainly some cells, mainly lymphocytes, are seriously damaged and destroyed by ionizing radiation, and other cells, such as macrophages, are delayed in their phagocytic function.

In particular, interference with metabolic activity (see Donaldson et al. [24]) in macrophages (very important cells in inducing antibody production, as indicated by Fishman and Adler [25], and recently tested by Gallily and Feldman [26]) can explain partial or total suppression of immune response in whole-body X-irradiated animals.

Benassi [27] suggests that DNA synthesis should not be stopped, within certain limits, by radiation in those cells in which such synthesis is already advanced; on the contrary, when radiation precedes antigenic stimulus, or follows it shortly afterwards, i.e. in the DNA presynthetic period, the subsequent process in antibody formation does not take place.

Lastly, another problem must be considered, i.e. the effects of radiation on delayed hypersensitivity, although until Taliaferro et al. [4] it was not associated with immunity to any infection. Delayed hypersensitivity is transferred not by serum, but by cells only, especially lymphocytes, Sell and Benacerraf [28]; it is manifested in vivo by the delayed skin reaction; it is present when antibodies of the conventional type are absent. The effects of radiation on delayed hypersensitivity are contradictory. Salvin and Smith [29] report that delayed hypersensitivity is not appreciably affected when antigen is injected from three days before to 18 hours after irradiation; similarly, Uhz and Scarff [30] find the delayed hypersensitivity persists when antigen is injected after irradiation. In contrast to the foregoing results Brooke [31] reports that delayed hypersensitivity is markedly depressed when antigen is injected on the same day, or 1, 7, 14 or 28 days after irradiation.

In helminthic infections the central interest, the problem of body irradiation, has been scarcely studied. Ionizing radiation has been given in vivo to animals to affect vitality, capacity of entering, development and maturity of helminthic parasites in the bodies of hosts previously, or not previously, infected.

Tyzzer and Honeij [32] irradiated (by radium) rats preinfected by larvae of *Trichina spiralis*. Irradiation with a tube of 8.8 mCi strength from the second day following the ingestion of larvae and with an additional tube containing 6.4 mCi five days after infection resulted in retardation of development, as shown by the number of immature females; 32% of these showed no fully-formed embryos, compared with 2% in the control animals.

Radiation given with a tube of 11.8 Ci strength from the time of the ingestion of larvae appeared to be even more effective; in fact, very few females, none of which contained embryos, were found at necroscopy at the end of seven days.

Radium radiation of mice from the interior of the intestine - employing a minute glass tube of emanation - in amounts sufficient to cause the death of these animals, failed to prevent the development of *Trichina spiralis*, as shown at necroscopy five and six days after the ingestion of larvae.

Semrad [33] gave 1000, 1500, 2000, and 2250 R in a daily exposure of 250 R to rats infected with 1000 larvae of *Trichina spiralis* one day before the initial exposure to X-radiation. He reported a reduction in the number of larvae encysted in the diaphragms of irradiated animals; the cumulative effects of the rays were evidenced by the fact that the severity of infection decreased inversely with increase in the dose of radiation.

With 800 R Honess [34] irradiated rats previously infected with 150 trichinae. After 30 days the rats were killed. They showed a reduction of about 26% in the number of trichinae in the diaphragm compared with control animals.

Duxbury and Sadun [35] irradiated *Anopheles quadrimaculatus* with increasing doses of ^{60}Co (1000, 2000, 4000, 10 000, 20 000, 40 000 rep)

24 hours after infection with larvae of Dirofilaria uniformis. They reported a marked reduction in the number and inhibition in the normal growth of helminthic larvae in mosquitoes irradiated with 4000 rep and more.

Villella et al. [36] carried out research on insects (Coleoptera) previously infected with embryonated eggs of Hymenolepis diminuta, and afterwards irradiated them with 18 000 R ^{60}Co. The authors obtained cysticercoids maturing in the rat with difficulty, and in a small number, compared with controls.

Stoner and Hale [37], Dunsmore [38], Yarinsky [39, 40], Connan [41], Coleman et al. [42], and Duxbury and Sadun [35], carried out research on hosts irradiated before infection.

Stoner and Hale [37] irradiated three groups of mice one to three hours prior to infection with Trichina spiralis with 550, 580 and 650 rep, respectively, and reported in these three groups – compared with non-irradiated control mice and irradiated but non-infected control mice – a high death rate which increased in proportion to the increase of infection and radiation dose; that testified, according to the authors, for an increased susceptibility of pre-irradiated mice to infection with Trichina spiralis (it must be noted that these authors, apart from one, did not study other possible causes of death – for example, eventual post-irradiation endogenous bacteraemia).

Yarinsky [39] selected four groups, subdivided into four subgroups, of mice and exposed them to 250, 350, 450 and 650 R, respectively, in a single dose. Three of four subgroups were infected orally with 50, 100 and 200 larvae of Trichina spiralis; the mice of the fourth subgroup (control) were not infected.

In a period of 28 days, following irradiation and infection, the author noted in the mice exposed to 650 R, and infected, a proportion of deaths significantly larger than that observed in the control subgroup. Radiation exposures of 250 and 350 R, followed by the various infections, did not result in host mortality throughout the period of observation. In other research, Yarinsky [40] observed, in mice infected with 200 larvae of Trichina spiralis, a higher number of adult parasites in mice irradiated with stronger doses (450 and 650 R) and killed after a longer period.

Dunsmore [38] exposed sheep to a dose of 440 rad 24 hours before direct injection into the rumen of Ostertagia 3rd-stage larvae. The sheep were killed 14 days after the larval administration and the author observed that the percentage of larvae showing arrested development (result of immune response, according to the author) was 18.7%, while in the controls infected, but non-irradiated, the percentage of arrested development was 43.2%.

Connan [41] irradiated guinea-pigs at 500 R one hour prior to receiving 2000 3rd-stage larvae of Trichostrongylus colubriformis and pointed out that the larvae did not migrate into the posterior part of the intestine, compared with non-irradiated control animals. The author suggested that radiation inhibited the normal immune response, which forced the larvae to migrate – after some time – from the anterior to the posterior part of the intestine.

Coleman et al. [42] locally irradiated the ileum, surgically isolated, of one group of mice, with 650 R, the body being shielded. They exposed a second group of mice, with the ileum shielded, to 550 R, and subjected a third group of mice to 550 R whole-body irradiation. Adult specimens of Hymenolepis nana were surgically implanted in the ileum of X-irradiated animals 24 hours after exposure.

Results showed that suppression of the immune response occurred in the second and third groups of mice; vice versa, local irradiation of the ileum, with the body shielded, did not inhibit antibody formation.

Duxbury and Sadun [35] exposed Anopheles quadrimaculatus to different doses of γ-rays from ^{60}Co 24 hours prior to infection with larvae of Dirofilaria uniformis and found statistically significant higher numbers of larvae in mosquitos exposed to 10 000 rep, compared with non-irradiated infected controls.

Moskwa et al. [43] carried out research on hosts exposed to irradiation before and after infection. The authors exposed rats, infected with 340 to 400 larvae of Trichina spiralis, to small doses of radiation before and after infection (100 R three days before, 50 R after one week, 50 R after two weeks) and did not observe differences in the number of encysted larvae in irradiated and infected rats, compared with non-irradiated infected controls.

Finally, a limited group of researchers used radiation treatment in animals previously immunized.

Stoner and Hale [37] irradiated with 600 rep ^{60}Co mice previously immunized against Trichina spiralis with sublethal infections, and followed by a challenge dose of 1500 larvae. There were no survivors in mice immunized, irradiated and challenged, but 97.5% of control animals irradiated but not immunized and not challenged, survived; 74% of the control, immunized and challenged, but non-irradiated animals, did not die.

Larsh et al. [44] irradiated (450 R) various groups of mice immunized against Trichina spiralis, 4, 8 and 12 days before a challenge dose of 200 larvae. Seven days after challenge the animals, killed, showed that irradiation produced complete or incomplete loss of acquired immunity. In fact, histo-pathological pictures in these mice (compared with those in control animals, some immunized and challenged, but non-irradiated, some only challenged) showed damage to the haematopoietic system and scarce cellular exudation in the intestine. The intestinal inflammatory response would be, together with serological factors, an expression of immunity in this parasitic disease, according to the authors.

The experiments of Yarinsky [40], who exposed mice immunized against Trichina spiralis to X-irradiation at different times prior to challenge are similar. The author noted, in animals irradiated with 450 R eight days before a challenge infection of 200 Trichina spiralis larvae, a complete loss of immunity manifested by equal numbers of adult parasites found in mice immunized, irradiated and challenged and in mice subjected only to challenge infection, together with a heavy reduction of circulating neutrophils and lymphocytes.

Ciordia and Jones [45] observed that rats previously immunized against Hydatigera taeniaeformis could be infected after being subjected to 1250 R of γ-irradiation (^{60}Co).

Trawinsky [46] X-irradiated (with high doses) sheep (600 R) and rabbits (400 R), artificially immunized with parasitic extracts (C. cellulosae, Trichina spiralis, and Distoma hepaticum) – and hence positive to the precipitate in reaction – and observed that the precipitate in reaction became negative for some time.

DISCUSSION

The results reported in the literature testify, in non-immunized animals irradiated at the time of infection, or not long after infection, the establishment of a resistance to infection, proved in the case of trichinosis, by a reduction in the number of Trichina larvae in muscle in irradiated animals, compared with non-irradiated controls.

It is possible that these results in trichinosis are altered by the direct action of ionizing radiation on the gonads of Trichina spiralis in the intestine; this suspicion, put forward by Taliaferro and Taliaferro, criticizing the work of Semrad, appears well grounded in the experiment of Tyzzer and Honeij [32].

Vice versa, the results obtained in those experiments in which infection followed irradiation, Stoner and Hale [37], Dunsmore [38], Yarinsky [39], Connan [41], are certainly more reliable.

Heavier infection is seen in animals which were irradiated by strong doses prior to the infection. This is shown by: more frequent mortality in hosts, Stoner and Hale [37], a larger number of parasites in localization, Yarinsky [39], a lower percentage of larvae showing arrested development, Dunsmore [38], and suppression of serological immune response, Coleman et al. [42]. When irradiation is administered locally, however, the antibody formation is not inhibited, Coleman et al. [42].

The results obtained by Moskwa et al. [43] are difficult to explain because of the superimposition of radiation doses administered before and after a trichinic infection.

The results reported in animals, previously immunized, irradiated and tested, after irradiation, by antibody titre or by challenge infection are interesting. These results show incomplete or complete loss of acquired immunity following strong doses of radiation, loss proved by: the equal numbers of parasites in the intestines of immunized, irradiated and challenged animals and in animals subjected to challenge infection only, Yarinsky [40], reinfection in immunized and irradiated animals, Ciordia and Jones [45], and negative precipitate in reaction, Trawinsky [46].

Lastly, in helminthic infections, delayed hypersensitivity has been studied in connection with total-body irradiation.

Kim [47] has shown that the delayed hypersensitivity to Trichina spiralis antigen in guinea-pig is not inhibited by total-body X-irradiation up to 200 R when administered 18 hours prior to sensitization with antigen plus adjuvant. An X-ray dose of 200 R, however, seems to inhibit it partially.

A particular aspect of body irradiation is the local administration for therapeutic purpose. In the literature there are few reports on this subject. Among them are: X-irradiation with this object, in man and dog affected by filariasis, Jaffe [48], Galliard [49]; therapeutic attempts, carried out by Sava [50] on dogs affected by demodectic scabies; experiments of Galliard [51] on the X-irradiation of dogs infected with Strongyloides stercoralis; experiments of Branscheid [52] and Marques et al. [53] on the administration of ionizing radiation to patients with hydatidosis; and studies of Merle [54] and Puget et al. [55] on roentgen therapy of so-called "summer sores" in horses.

The known data are not sufficient to understand how much in this phenomenon is concerned with ionizing radiation per se (i.e. direct action on the parasite or tissues in an anti-inflammatory sense) and how much is the concern of immune processes.

Further studies are consequently essential for the explanation of this problem: study of the exact mechanism of the increased antibody level in X-irradiated animals under particular conditions included (see suppositions and research of White and Dougherty [56], Rigos [57], Koga [58], Benassi [27], Loiseleur [59], etc.).

AUTHOR'S INVESTIGATIONS

The problem of experimental infection by Ascaris suum in guinea-pigs exposed to total-body X-irradiation was studied.

Preliminary research convinced the author that more significant data could be obtained in animals, such as guinea-pigs, highly radiosensitive (see Trum and Rust [3]) and very susceptible to this microascariasis. Therefore, 40-day-old guinea-pigs weighing 200 - 250 g, haematologically homogeneous, were used and were infected (some four hours and some four days after irradiation) with a single dose abundantly lower than lethal (1500 embryonated eggs by oral administration).

The animals (males only) were never subjected to protective treatment against ionizing radiation nor against migrating larvae, to eliminate every interference in the experiment.

In the first part of the research 120 guinea-pigs, divided into four groups, were used.

Group I

Thirty guinea-pigs were subjected to total-body X-irradiation with $LD_{50/30}$. Ionizing radiation was given in a single dose in relation to the weight, using a Costantix Rangoni and Policelli X-ray machine with 0.25 mm copper and 1.0 mm aluminium filters at a target distance of 80 cm* and four hours after irradiation the guinea-pigs were infected with 1500 embryonated eggs of Ascaris suum by oral administration.

Group II

Thirty guinea-pigs were irradiated in the above-mentioned manner and, four days after irradiation, infected orally with 1500 embryonated eggs of A. suum.

Group III

Thirty guinea-pigs were merely irradiated in the same manner (irradiated controls).

Group IV

Thirty guinea-pigs were subjected to ascaridic infection only with 1500 embryonated eggs of A. suum given orally (infected controls).

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* This machine was placed at the author's disposal by Camen, San Piero, Pisa.

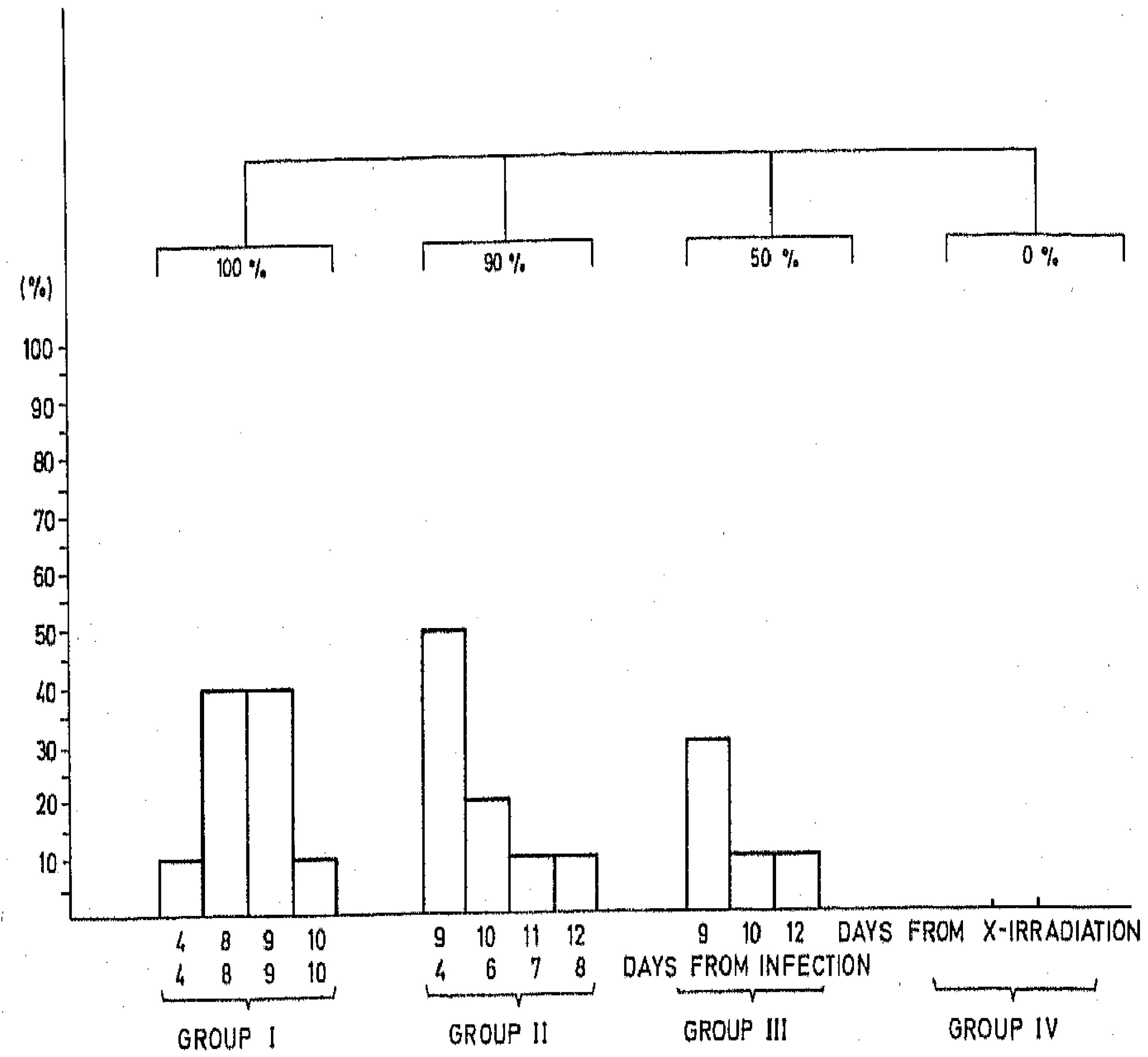

FIG.1. Percentage of dead animals at 30th day after X-irradiation.

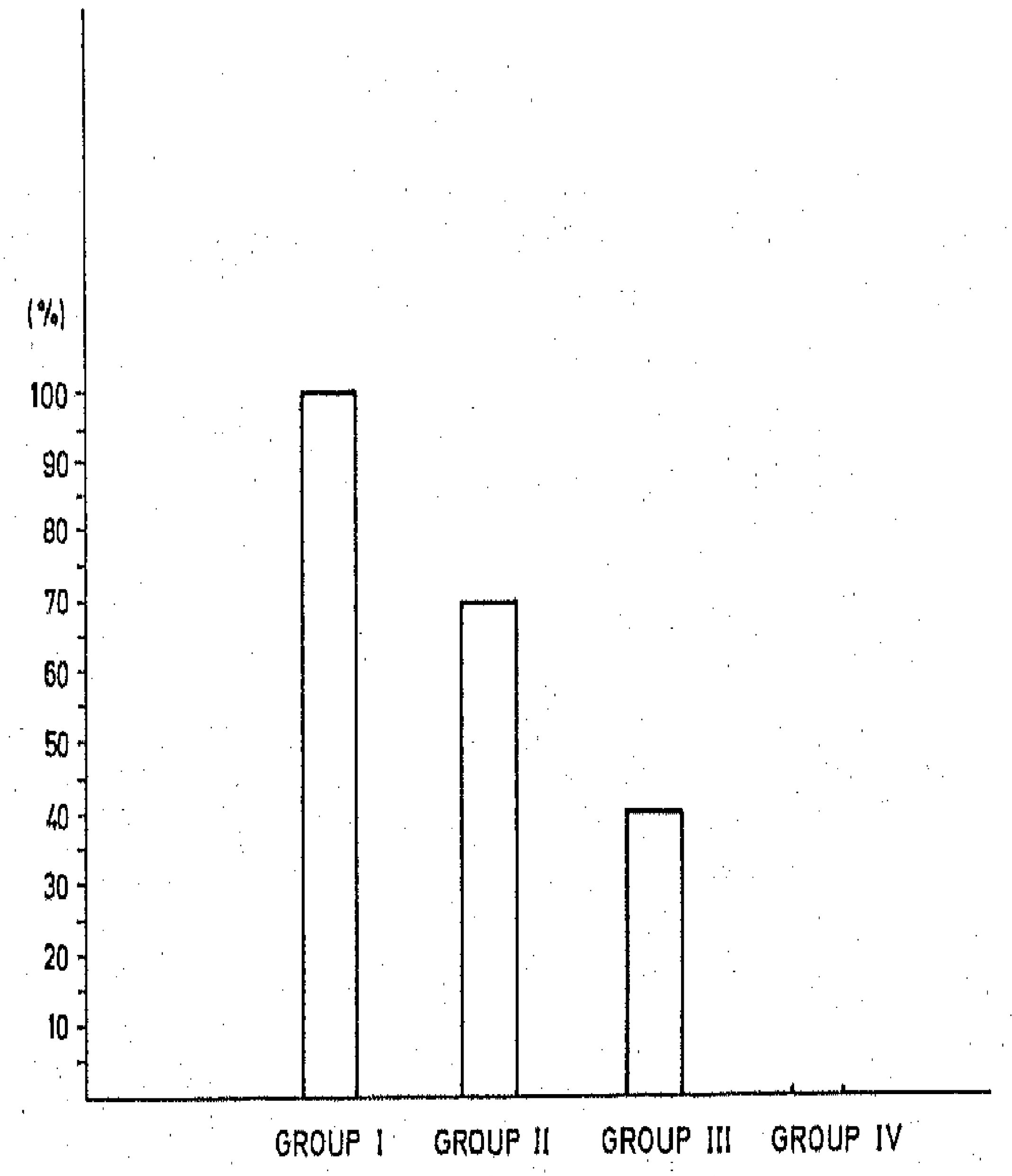

FIG.2. Percentage of dead animals at 10th day after X-irradiation.

Predecease was studied in groups I and II in comparison with the control groups III and IV. The data are summarized in Fig. 1.

Animals, infected four hours and four days after X-irradiation, predeceased, compared with irradiated controls; the peak of deaths was

between the eighth and the ninth day after infection (eight and nine days after irradiation) for group I, and between the fourth and sixth day after infection (nine and ten days after irradiation) for group II. Ten days from the beginning of the experiment 100% of the animals died in group I and 70% in group II (Fig. 2). On the 12th day the percentage of dead animals increased to 90% in group II. This situation persisted until the 30th day, that is, the period in which the predecease was 50% for group III, according to expectations, and there was none in group IV (see Fig. 1).

Clinically, in the days preceding death, the animals in groups I and II showed dysorexia, rapid thinning, anaemia, tachycardia and increasing dyspnoea. Not long before death the animals showed extreme prostration and hypothermia and died almost always with haemoptysis.

Anatomo-pathological examination revealed severe anaemia of skin and oculo-conjunctival, nasal, oral and other mucosae. Non-coagulated blood was present in nasal cavities and on nostrils. Skeletal musculature appeared hytrophic.

Haemorrhagic points or spots were seen in the peritoneum, liver, kidney and suprarenal bodies. The intestine, mainly the small, showed spot-like or diffuse haemorrhages. A similar picture was observed in the stomach, where haemorrhagic foci in the form of blood clots or haemorrhagic spreading were marked sometimes by lenticular ulcera transparent on the outer surface of this organ.

Severe pulmonary haemorrhages were present in the thoracic cavity, together with pericardial and epicardial haemorrhages. The lungs, red-brown in colour, showed brown-blackish miliary foci and spots. It was possible to see the haemorrhagic picture of the lung clearly on the section because this organ was soaked with blood. Partially, or non-coagulated blood was present mixed with air bubbles and mucus in the trachea and bronchi. On the surface and on the section of the brain haemorrhagic points were found.

At the histological examination there were found to be pulmonary lesions and plurivisceral haemorrhagic diathesis. Heavy soaking of blood was present in the alveoli, bronchioli, intra- and extra-lobular bronchi of dead animals of groups I and II. Fibrinous nets were absent in many areas; neutrophil leucocytes were very scarce. Occasionally, swelling of some desquamating parietal cells was seen in the alveoli. Small intra- and extra-lobular bronchi were marked by ectasic, thin walls and by the lumina full of blood. Vice versa, larger extra-lobular bronchi were only partially occluded by extravasated blood. Areas in which the soaking of blood in the alveoli was less severe were marked by some erythrocytes mixed with plasma material. Ascaris larvae (3rd and 4th stage) were seen here and there in parenchyma and in the bronchial lumina, mixed with blood; they were never sequestered by inflammatory reactions.

Stomach and intestine showed heavy haemorrhages and ulcerous areas in mucosa until submucosa. Haemorrhagic foci were also present in the myocardium, kidney and liver, together with degenerative processes.

Irradiated but non-infected controls that died during the same period as the above-mentioned guinea-pigs (i.e. from the sixth to the twelfth day after irradiation) showed similar clinical pictures without, however, evident dyspnoea and haemoptysis. The anatomo-pathological pictures were like those of groups I and II, except pulmonary haemorrhagic lesions, which were always limited to a few subpleural and deep haemorrhagic points, visible on the white-pink background of the organs.

Infected, but non-irradiated controls, did not predecease within 30 days of the experiment. After killing, at the necroscopy they showed the consequences of larval migration in the liver and lung.

In all irradiated and infected guinea-pigs larvae in the lung were extracted by the Baermann apparatus at 37°C. There was always an active pulmonary migration of Ascaris larvae.

In conclusion, the above-mentioned research showed a significant predecease of animals in groups I and II compared with groups III and IV (irradiated controls and infected controls, respectively).

Clinical, anatomo-pathological and histological pictures and parasitological findings suggested that predecease of irradiated and infected animals was due to severe haemorrhages in the lung caused by helminthic trauma, together with haematic dyscrasia by total-body X-irradiation.

It was considered advisable to carry out another experiment to study: (a) larval migration and the development of Ascaris suum; (b) haematic dyscrasia; (c) possible bacteraemia in guinea-pigs subjected to total-body X-irradiation.

Such research is progressing and preliminary results justify the hypothesis that predecease in irradiated and infected animals is due to severe pulmonary haemorrhages caused by numerous vascular traumatisms produced by migrating larvae in animals affected by severe haematic dyscrasia by radiation. In particular, larval migration in the liver and lung is similar in irradiated and infected animals and in animals merely infected, even if it occurs a little earlier in the first compared to the second.

Other research is in progress to study whether previous vaccination of guinea-pigs against Ascaris suum is able to hinder predecease.

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TABLE I. PLASMA ALBUMIN METABOLISM ON FLUKE-INFECTED AND NORMAL RABBITS

	Rabbits (No.)	Plasma albumin (g/100 ml)	Intravascular pool CA (g/kg)	Degradation rate Apparent $t_{\frac{1}{2}}$ (h)	Fractional K^a	Catabolic rate K^b
INFECTED	4	2.54 ± 0.25	0.99 ± 0.14	83 ± 16.6	0.431 ± 0.082	0.433 ± 0.073
CONTROL	4	3.38 ± 0.55	1.26 ± 0.26	211 ± 43.5	0.201 ± 0.071	0.191 ± 0.052

[a] Calculations as described by Campbell et al. [9].

[b] Fractional catabolic rate calculated according to Matthews [10].

corresponding total intravascular activity, Campbell et al. [9], and also from analysis of the plasma activity curve, Matthews [10]. The results of this experiment are shown in Table I.

It is clear from the results in Table I that no very useful information on the effects of liver fluke disease or plasma protein metabolism can be obtained by conventional analytical techniques. The concentration of albumin in the plasma and the total intravascular pool are only slightly lower in the infected than in the normal animals. On the other hand, the degradation rate of albumin is dramatically greater in the fluke-infected rabbits. It is worth noting that there is very good agreement between the values for K determined by Matthews' method and that of Campbell et al.

It has been suggested in the past that interference with albumin synthesis due to liver damage might be a contributory factor to the hypoalbuminaemia of fascioliasis. This certainly cannot be true of the eight- to ten-week infection in rabbits. That the hypercatabolism demonstrated in Table I does not give rise to a very severe hypoalbuminaemia is due to the fact that, so far, in these rabbits synthesis must have been almost keeping pace with breakdown, and thus proceeding at a rate in the infected animals much greater than that in the controls.

Whereas ^{131}I-labelled albumin is a very suitable material for the study of albumin turnover, it is not suitable for the direct measurement of protein loss into the gastrointestinal tract because of its breakdown there and the re-absorption of labelled fragments. Direct measurement of the movement of plasma macromolecules into the gut is however possible, using labelled polyvinylpyrrolidone (PVP).

The fæcal excretion of ^{131}I-labelled PVP in fluke-infected and normal rabbits

Iodine-131-labelled PVP was injected into five fluke-infected (50 metacerceriae each ten weeks earlier) and five control rabbits. The plasma level and the fæcal output of PVP were followed for a period of five days. Very significant differences between the infected and control animals, both in the plasma disappearance curve and in the fæcal output, were found.

The results are summarized in Table II. In the infected animals the plasma half-life of PVP was much shorter and the fæcal output very substantially greater than that of the controls, thus representing a significant movement of plasma macromolecules into the gut, presumably via the flukes and bile.

The results of this, and the previous experiment, are most easily explained in terms of direct loss of plasma protein into the gastrointestinal tract, presumably via flukes and bile.

Experiments with ^{51}Cr-labelled red cells

Four fluke-infected rabbits (100 metacercariae each ten weeks previously) and four controls were each injected with autologous red cells

TABLE II. PLASMA "HALF-LIFE" AND FÆCAL EXCRETION OF PVP IN FLUKE-INFECTED AND NORMAL RABBITS

	Rabbit (No.)	Half-life plasma PVP (h)	Cumulative (5 day) fæcal output as % of injected dose	Mean daily plasma "clearance" (fæcal) (ml)
INFECTED	971	36	17.0	27.2
	084	50	10.3	19.7
	008	39	12.9	28.3
	996	43	16.9	32.8
	336	40	20.3	40.6
CONTROL	985	88	2.1	2.3
	110	73	2.5	3.7
	199	65	4.0	4.7
	CLT	68	2.5	3.0
	203	60	4.1	5.1

labelled with ^{51}Cr. The persistence of labelled cells in the circulation and the urinary and fæcal output of isotope were followed over a period of eight days. The results are summarized in Fig. 1 and Table III.

Differences between the infected and normal animals are apparent in the blood disappearance curves, the infected animals showing a substantially shortened half-life for labelled cells, and also in the fæcal output of radioactivity. This is expressed in Table III as a 'fæcal clearance', i.e. the amount of blood which would have to leak into the gastrointestinal tract each 24 hours to account for the fæcal radioactivity. Again, there is a very striking increase in the fluke-infected animals. (The validity of the ^{51}Cr-labelled red cell technique for this type of measurement is discussed in another paper at this meeting[1]).

Taken together with the results obtained above with labelled plasma proteins and PVP, these findings further substantiate the idea of a whole blood loss caused by the parasite.

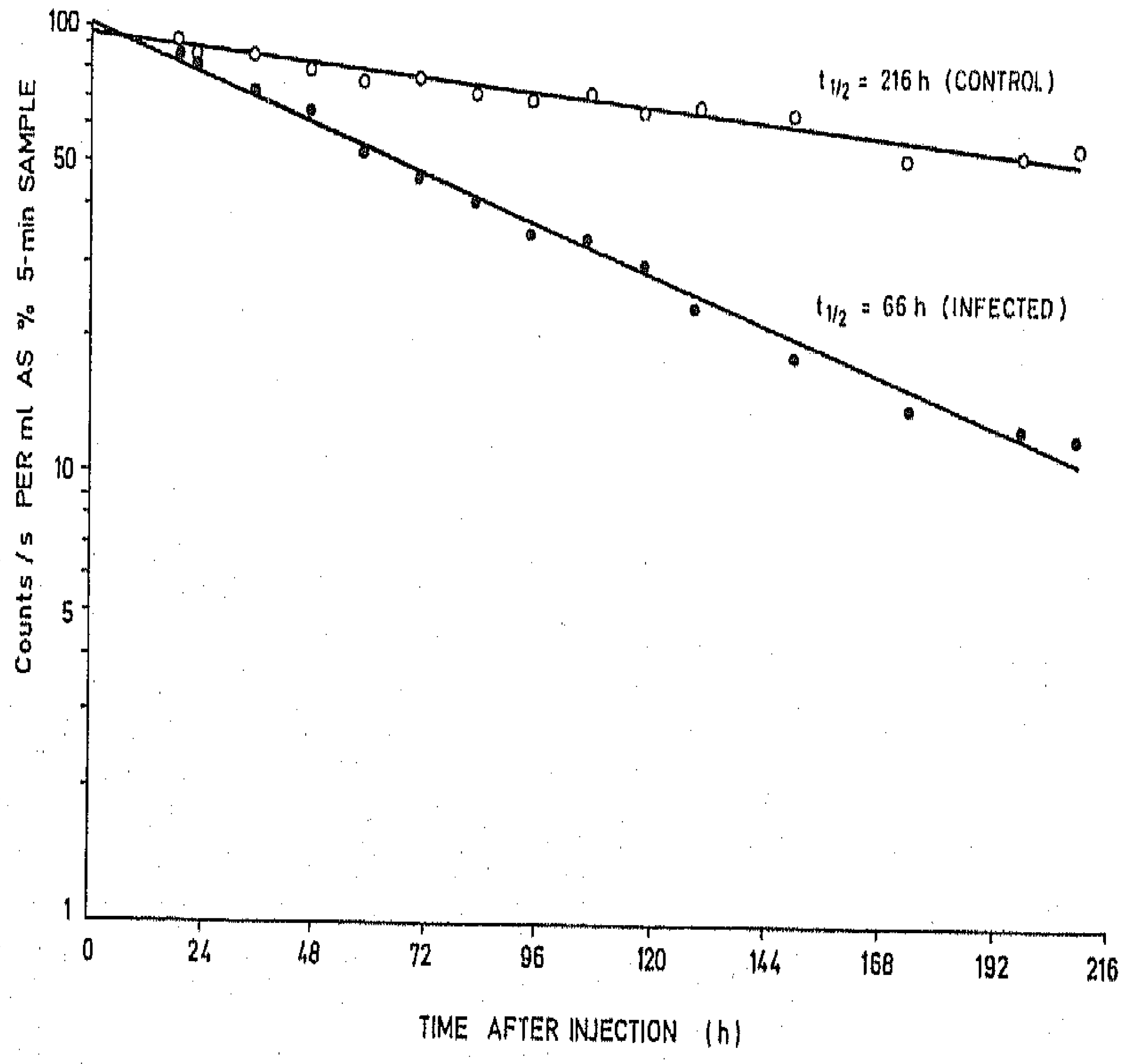

FIG. 1. Disappearance curve of ^{51}Cr-labelled red cells from the circulation of 'control' and 'fluke-infected' rabbits.

Simultaneous measurements with ^{51}Cr-labelled red cells and ^{95}Nb-labelled plasma protein

The isotopes ^{95}Nb and ^{51}Cr form a suitable pair for simultaneous measurement of protein and red cell loss in that they can be measured in the same sample because of differences in the energy spectrum of their radiations. Plasma albumin was labelled with ^{95}Nb by a modification of the method described by Jeejeebhoy, et al. [11]. Three fluke-infected and three control rabbits were injected with niobium-labelled albumin and chromium-labelled red cells at the same time. Over 24-hour periods

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[1] JENNINGS, F.W., "Anaemias associated with helminthic infections", these Panel Proceedings.

TABLE III. BLOOD LOSS IN FLUKE-INFECTED AND NORMAL RABBITS CALCULATED FROM FAECAL EXCRETION OF ^{51}Cr

Group	Rabbits (No.)	Mean faecal clearance	
		Whole blood (ml)	Red cells (ml)
INFECTED	1	26.0	5.6
	2	3.1	1.0
	3	15.2	3.4
	4	42.7	6.7
CONTROL	5	0.20	0.07
	6	0.22	0.07
	7	0.16	0.06
	8	0.23	0.09

fæcal output of ^{51}Cr was measured and 'blood clearance' was calculated; likewise, a 'plasma clearance' based on the 24-hour fæcal output of ^{95}Nb. These measurements were made daily for a total of 11 days and then both control and infected animals were treated with an anthelminthic (Zanil[2]- 0.5 ml/kg) and the measurements continued for a further period of nine days. The anthelminthic treatment was carried out to see if red cell and plasma loss stopped abruptly as soon as the flukes were killed. The results of this experiment are shown in Table IV.

The 'background' protein leak as manifested by the plasma clearance in normal animals, both pre- and post-treatment, and in the post-treatment period in infected animals, is much more substantial than the background blood loss. This is to be expected as a normal leakage of plasma protein into the gastrointestinal tract occurs in all animals. Even when allowance is made for this the plasma protein loss into the gut is clearly much greater than can be accounted for by the whole blood loss. It would appear therefore that liver-fluke infection causes a loss to the host of the whole blood, very probably due to the feeding activities of the parasite, and over and above this a loss of plasma protein, presumably occurring as a result of damage to the bile ducts.

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[2] Imperial Chemical Industries.

TABLE IV. FAECAL "BLOOD" (^{51}Cr) AND "PLASMA" (^{95}Nb-ALBUMIN) CLEARANCE IN FLUKE-INFECTED AND NORMAL RABBITS BEFORE AND AFTER ANTHELMINTHIC TREATMENT

	Rabbit (No.)	Pre-treatment		Post-treatment	
		Mean "blood" clearance (ml)	Mean "plasma" clearance (ml)	Mean "blood" clearance (ml)	Mean "plasma" clearance (ml)
INFECTED	036	16.8	25.1	1.0	5.5
	182	5.8	18.7	0.8	5.5
	712	5.9	37.0	2.0	4.1
	Mean	9.5	23.6	1.3	5.0
CONTROL	027	0.07	4.19	0.11	3.67
	384	0.13	10.4	0.09	14.8
	X	0.11	[a]	0.44	[a]
	Mean	0.10	7.3	0.21	9.2

[a] ^{95}Nb activity too low for accurate measurement.

DISCUSSION

In attempting to determine whether or not the liver fluke is essentially haematophagic two approaches are possible. One may label the cells or plasma proteins of the host animal and by analysis of the liver flukes attempt to calculate the amount of blood which they have consumed (cf. Jennings et al. [4]). The difficulty in this type of experiment is that of assessing to what extent the radioactivity of the flukes is due to the uptake from the bile of labelled breakdown products of either the cells or the plasma proteins. In spite of this, the magnitude of the activity found in the flukes will provide strong suggestive evidence about their feeding habits.

Results obtained from this type of experiment cannot be regarded as providing precise quantitative information on the loss of red cells or plasma proteins from the host.

Essentially similar to the direct isotopic methods described here is the approach of Todd and Ross [12], who analysed the caecal contents of liver flukes and concluded that they contained breakdown products of the host's haemoglobin.

The alternative to direct analysis of liver flukes and a preferable method from the point of view of assessing the pathogenic effects of the parasite is to study the metabolism of red cells and plasma protein in the host animal and to see if the changes observed in infected animals are consistent with a blood loss brought about by the parasite. This is the approach which is represented in the experiments described above.

Hypercatabolism of albumin, increased fæcal excretion of injected PVP, shortened half-life of ^{51}Cr-labelled cells, with the concomitant increase in fæcal output of ^{51}Cr, all occur in the infected animals and are consistent with blood letting brought about by the flukes. The experiments with niobium-labelled protein and chromium-labelled cells further indicate a loss of plasma protein over and above that accounted for in the blood loss. This is explicable in terms of the structural changes which can be seen in the bile ducts of infected animals (Murray and Jarret [13]).

It must be made clear that all the experiments described above were carried out on animals with nine- to ten-week infections, i.e. they were harbouring a population of fully mature parasites. It will be necessary to study animals during the early stages of the disease, e.g. when the flukes are migrating through the liver, and also animals with long-standing infections, to find out whether the straightforward blood-loss explanation is not further complicated in these instances by impairment of synthetic processes, i.e. impairment of protein synthesis and/or haemopoiesis.

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HYPOPROTEINAEMIAS ASSOCIATED WITH HELMINTHIC INFECTIONS, WITH SPECIAL REFERENCE TO GASTRO-INTESTINAL PARASITES

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Abstract

HYPOPROTEINAEMIAS ASSOCIATED WITH HELMINTHIC INFECTIONS, WITH SPECIAL REFERENCE TO GASTRO-INTESTINAL PARASITES. The syndrome of protein-losing gastroenteropathy in man is briefly outlined, with special reference to the detection of an abnormal enteric loss of serum proteins by means of labelled macromolecules, e.g. ^{131}I polyvinylpyrrolidone, ^{51}Cr albumin, ^{67}Cu coeruloplasmin, ^{59}Fe iron-dextran and ^{131}I- or ^{125}I-labelled serum proteins. These methods have not been extensively used in veterinary research with large animals.

Several diseases of domestic animals are rather close analogues to human disorders in which a gastrointestinal protein loss has been demonstrated. Hyperplastic gastritis in cattle and swine (caused by *Ostertagia ostertagi* and *Hyostrongylus rubidus*) are examples of such analogues. Others are regional enteritis and ulcerative colitis in pigs. Studies of these diseases have revealed that a gastrointestinal protein loss is an essential factor in the development of pronounced hypoproteinaemia. The probable site of this enteric protein loss is the dilated villous lymphatics (chylus ampoules) that are seen throughout the small intestine during chronic diarrhoeal conditions of cattle and swine. However, the available evidence indicates that the loss of serum proteins via the digestive tract in animals is not *solely* related to the presence of lymphangiectasias or to other morphological lesions. Diarrhoea per se seems to be an important factor in triggering the enteric protein loss. This theory is sustained by the finding that hypercatabolism (due to the gastrointestinal loss) is restricted to diarrhoeal phases of the disease, whereas hypocatabolism follows when the animal enters a quiescent phase or recovers. Accordingly, studies of the basic aspects of diarrhoea appear to be very important.'

During the last decade much progress has been achieved in studies of the ecological, immunological and therapeutical aspects of intestinal parasitism. Studies on the pathogenesis of *secondary* manifestations of parasitic gastroenteritis have been more scarce. One such secondary manifestation is the severe hypoproteinaemia that invariably follows chronic infections with trichostrongylides in ruminants. Recently, this symptom has attracted considerable interest in the light of the discovery of gastrointestinal protein loss in certain diseases of man. This important progress was initiated in 1957 when Citrin and co-workers [1] found that a human patient suffering from Menetriér's disease (hypertrophic gastritis) excreted enormous amounts of serum albumin into the stomach, which gave evidence that the pathogenesis of the severe hypoalbuminaemia seen in this disease was connected with a "leakage" in the hypertrophic mucosa, permitting the passage of macromolecules into the lumen. The observation of Citrin and co-workers has been repeatedly confirmed by other authors (see e.g. Schwartz and Jarnum [2], Jeffries and Sleisinger [3]) and up till the present time the syndrome of gastrointestinal protein loss has been demonstrated in a variety of diseases, e.g. cancer of the stomach, regional enteritis, ulcerative colitis, tropical and non-tropical sprue, lymphogranulomatosis, Hirschsprung's disease and many others. The advent of this important progress in our understanding of hypoproteinaemic conditions was mediated by the introduction of radioisotopes into clinical medicine. ^{131}I-labelled serum proteins have been used extensively in the study of gastrointestinal protein loss of man. However, since proteins excreted into the lumen of the digestive tract are digested by bacterial and enzymatic activities, the detection of gastrointestinal protein loss is dependent upon special methods, some of which are briefly outlined below.

(a) Paper electrophoresis and immunoelectrophoresis of gastric and intestinal juice have been employed. However, these methods are not reliable due to hydrolysis of the excreted proteins, incomplete collections, methodological errors and the risk of admixture of blood to the secretions due to lesions caused by the tube. In ruminants, secretions from the abomasum and intestine cannot be sampled with a tube. In abomasal secretions obtained immediately after death serum proteins (especially albumin) were detected in cases of hyperplastic abomasitis (ostertagiasis) in cattle, Nielsen [4]. Electrophoretic methods in the detection of gastrointestinal protein loss are not widely used now that it has been shown that serum proteins are present in normal gastrointestinal secretions, the digestive tract being an important site of normal catabolism of albumin and several other serum proteins.

(b) The ^{131}I-PVP test was introduced by Gordon [5]. The polymer, polyvinylpyrrolidone (PVP) has an average molecular weight of 40 000 (range 20 000 - 75 000). It is not broken down in the digestive tract, nor is it absorbed to any significant degree. The bulk of ^{131}I-PVP excreted from blood to lumen of the bowel will, therefore, appear in the stools and the fæcal PVP excretion will indicate the degree of gastro-intestinal protein "leakage". Although PVP chemically, physically and biologically, is extremely different from serum proteins, the PVP test has been successfully used in diagnostic gastroenterology. In cattle suffering from ostertagiasis, Mulligan et al. [6] found high fæcal excretions of ^{131}I-PVP.

A similar method, using ^{59}Fe-labelled iron-dextran (Imferon) has been proposed recently by Andersen and Jarnum [7].

(c) ^{51}Cr-labelled albumin was introduced by Waldmann [8]. The test is based upon the fact that chromium is poorly absorbed from the gastrointestinal tract. This means that the label will appear in the stools after digestion of the labelled albumin. The most obvious disadvantage of this method is that slight denaturation of the preparations seems inevitable. This denaturation is reflected by very short plasma half-lives of ^{51}Cr albumin in normal subjects. This fact has encouraged the search for more suitable tracers for this specific purpose. One such tracer is ^{67}Cu, which may be used as a label for the copper-containing serum protein, coeruloplasmin. Copper-67-coeruloplasmin kinetics have been examined by Waldmann and Wochner [9], who found that ^{67}Cu coeruloplasmin had similar metabolic properties to ^{131}I coeruloplasmin. Accordingly, this preparation may prove useful in the study of gastrointestinal protein loss.

A common feature of the methods mentioned above is that they have been used very little in research work with large animals. Studies on gastrointestinal protein loss in cattle and swine have been conducted mostly with ^{131}I-labelled proteins, Cornelius et al. [10], Mulligan et al. [6], Halliday et al. [11] (personal communication), Nielsen [12, 13]. It has been shown in man that the fæcal excretion of ^{131}I is elevated in conditions with a gastrointestinal protein loss, even if no measures are taken to prevent re-absorption of the label, Jarnum [14]. This is true also in cattle and swine, Nielsen [12, 13] (cf. Table I). Although the fæcal excretion of ^{131}I is not a strictly quantitative measure of the degree of gastrointestinal protein loss - some of the isotope being absorbed from the intestine and excreted via the kidney - most authors have preferred ^{131}I-labelled proteins because such experiments allow calculations of all relevant turnover data (plasma volume, half-life, pool sizes, etc.). Other iodine isotopes are applicable as well, e.g. ^{125}I.

TABLE I. FAECAL EXCRETION OF ^{131}I IN CATTLE AND SWINE WITH GASTROINTESTINAL DISORDERS AFTER INTRAVENOUS INJECTIONS OF ^{131}I ALBUMIN. NO MEASURES WERE TAKEN TO PREVENT REABSORPTION OF THE ISOTOPE

	Subject	Diagnosis	Faecal excretion of radioactivity during first 4 days of experiment (% dose)
Cattle			
	170	Verminous abomasitis (diarrhoea)	16.2
	229	Johne's disease (diarrhoea)	9.1
	169	Verminous abomasitis (normal faeces)	2.8
	228	Johne's disease (normal faeces)	2.0
	Five normal controls		2.3 - 4.7 (mean: 3.5)
Swine	301	Regional enteritis (diarrhoea)	4.8
	68	ditto	8.1
	69	ditto	7.7
	Two normal controls		1.9 and 2.9

Turnover of serum proteins in parasitized cattle and swine

In northern Europe ostertagiasis in cattle appears to be increasingly important. The biology of Ostertagia ostertagi - the medium stomach worm - has been studied in detail by Scottish workers, who found that this

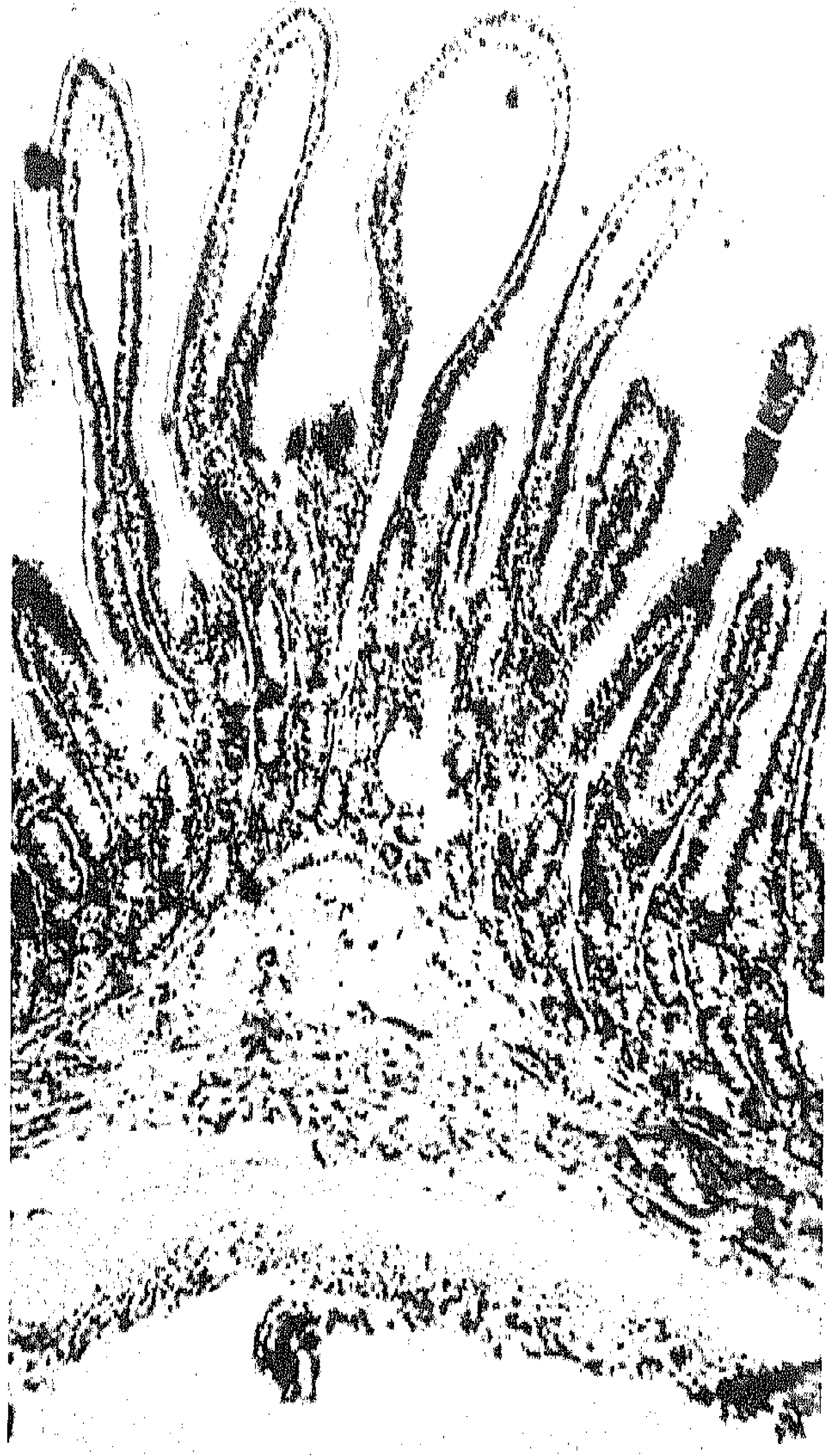

FIG.1. Jejunal mucosa from heifer with type-II ostertagiasis. Note dilatation of chylus ampoules.

parasite has several interesting characteristics, e.g. clinical ostertagiasis may be seen in housed cattle during the winter months, Martin et al. [15]. This peculiar seasonal incidence may be associated with inhibition of larvae ingested during late autumn, Armour and Urquhart [16]. These inhibited larvae may remain dormant in the abomasal mucosa for several months. The main clinical finding in chronic ostertagiasis is diarrhoea with emaciation. Necropsy reveals chronic hyperplastic abomasitis and catarrhal, atrophic enteritis. Histology of the small intestine reveals widespread abnormalities of the intestinal lymphatics. The chylus ampoules are dilated and distorted, thereby causing club-like distension of villi intestinales (Fig.1, from Nielsen and Andersen [17]). These lesions are very similar to the intestinal lymphangiectasia described in man by Waldmann et al. [18].

TABLE II. ^{131}I-ALBUMIN TURNOVER DATA IN CATTLE WITH CHRONIC ABOMASOENTERITIS

Animals examined during a period with profuse diarrhoea		Fractional turnover rate (K_{12}) (%/day)	Plasma half-life (days)
Subject			
170	Verminous abomasitis	15.5	11.0
129	ditto	13.8	10.9
153	ditto	17.6	8.0
447	Johne's disease	9.0	10.9
125	Abomasoenteritis catarrh. (1st experiment)	10.9	14.2
135	Verminous abomasitis	9.9	11.5
153	Abomasoenteritis catarrh.	8.1	14.8
201	ditto	10.9	15.0
202	Verminous abomasitis	11.0	10.9
424	Johne's disease	10.7	11.0
425	ditto	17.4	6.7
	Mean	12.3	11.4
Animals examined during a quiescent phase or during convalenscence:			
169	Verminous abomasitis	6.5	20.1
130	ditto	5.6	22.0
228	Johne's disease	4.3	34.0
229	ditto	5.3	27.0
125	Abomasoenteritis catarrh. (2nd experiment, convalescence)	3.6	48.0
140	Abomasoenteritis catarrh.	7.6	19.0
172	ditto	6.5	20.1
210	Johne's disease	6.9	19.6
	Mean	5.8	26.2
Normal controls	Mean	8.2	20.6
	Range	6.4 - 10.0	16.5 - 28.0

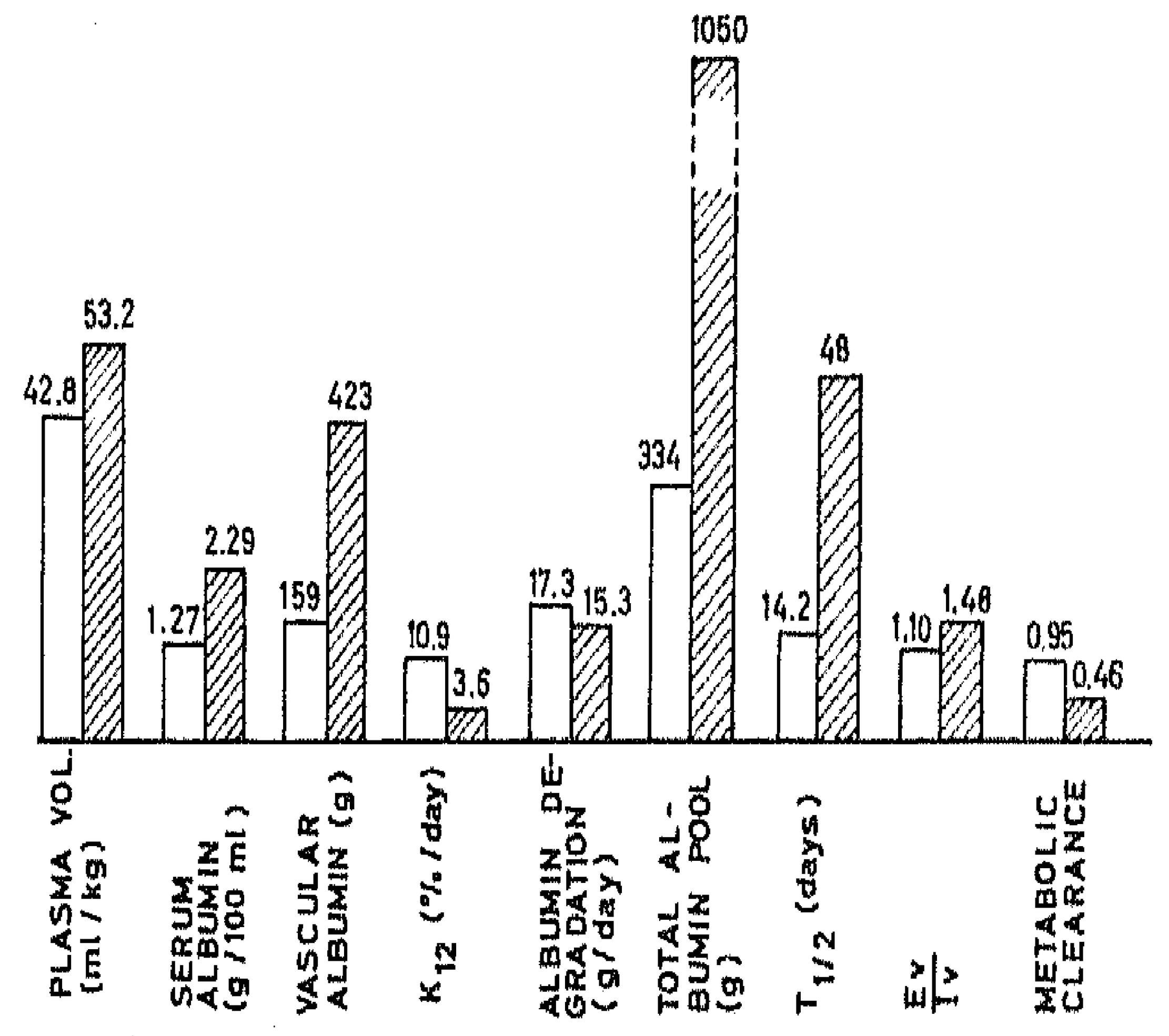

FIG. 2. ^{131}I albumin turnover data in heifer with chronic abomasoenteritis. The animal was studied during a diarrhoeal phase (white columns). Note relative hypercatabolism during diarrhoeal phase (high K_{12}, short plasma half-life) and hypocatabolism during remission (low K_{12}, long plasma half-life). The absolute degradation rate (g/kg per day) is nearly the same during the two phases of the disease in spite of the very small vascular albumin pool during the diarrhoeal phase.

A prominent biochemical finding in chronic ostertagiasis is severe hypoproteinaemia, the albumin fraction being most markedly depressed. Gastrointestinal loss of serum proteins is an important factor in the pathogenesis of this hypoproteinaemia. In this, the hyperplastic abomasitis in cattle would appear to be analogous with hypertrophic gastritis (Menetriér) in man. However, it has been shown that the enteric protein loss in cattle with ostertagiasis is restricted to the diarrhoeal phases of the disease. Cattle examined during diarrhoeal phases are hypercatabolic, with short plasma albumin half-lives, high fractional turnover rates and high fæcal excretion of ^{131}I. During quiescent phases, however, the turnover data indicate a hypocatabolic condition, with normal plasma albumin half-lives, normal or low fractional turnover rates and normal fæcal excretion of ^{131}I, Nielsen [12]. It has been postulated that the protein loss in hypertrophic gastritis of man might be associated with the enlargement of mucosal surface area. In cattle, at least, the protein loss is related to the clinical picture of the disease rather than to the gross pathological lesions of the abomasum (hyperplasia). This finding is sustained by preliminary results obtained in two sows with Hyostrongylus rubidus infections, Nielsen [13]. The pathological lesions of the stomachs in these sows were very similar to the Ostertagia lesions in cattle. However, the sows did not scour, the main clinical findings being emaciation - in spite of fairly good appetite - and occasional vomiting. Serum protein levels were normal, and ^{131}I-albumin turnover data did not indicate a gastrointestinal loss of serum proteins.

Table II summarizes the author's findings in 19 bovine patients with various gastrointestinal diseases (ostertagiasis, Johne's disease and chronic, catarrhal abomasoenteritis). It appears that the pattern of ^{131}I-albumin turnover is related to the clinical phases of the diseases examined and not to the nature or site of the actual macroscopic lesions of the gastrointestinal tract. In this respect it is important to note that a substantial gastrointestinal serum protein loss may be seen in simple catarrhal abomasoenteritis and, moreover, that this loss ceases when the animal enters a quiescent phase of the disease or recovers (Fig. 2). The intestinal lymphangiectasias described above (cf. Fig. 1) are seen in all these conditions; they seem to occupy most of the small intestine, even in abomasal disorders, and they may be very important sites of the intestinal transfer of serum proteins. Accordingly, the "leakage" of proteins in cattle is probably not confined to the diseased organ - e.g. the hyperplastic abomasum - but must be assumed to occur through the entire length of the small intestine. Several interesting features adhere to intestinal lymphangiectasia in cattle. Thus, the lesion seems to be reversible; it disappears if the primary disease is cured. Also, preliminary observations in the author's laboratory indicate that the mere presence of intestinal lymphangiectasia is not in itself accompanied by a gastrointestinal protein loss. A rapid intestinal transit of ingesta - i.e. diarrhoea - seems to be an important precipitating factor in the pathogenesis of the intestinal protein transfer. This emphasizes the need for research into the basic etiology, pathogenesis and effects of the symptom, diarrhoea. Interesting studies by Jennings et al. [19] indicate that the diarrhoea seen in ostertagiasis is not primarily caused by the worms but by increased bacterial growth in the abomasum as the pH rises from approximately 2 to approximately 7, a rise that is associated with loss of functional parietal cells during the early stages of infection. Also, mention should be given to the fascinating findings in man that diarrhoea may be associated with deficiencies of intestinal enzymes, e.g. disaccharidases, Littman and Hammond [20]. The significance of this and other factors in diarrhoeal disorders of cattle and other domestic animals remains to be assessed.

The survey given above has dealt mainly with ^{131}I-albumin studies. However, it has been shown that immunoglobulin G behaves essentially like albumin in diarrhoeal disorders of cattle and swine, Nielsen and Nansen [21]. A gastrointestinal immunoglobulin loss may prove very important in the pathogenesis of secondary bacterial infections which are frequently seen in diarrhoeal disorders (e.g. pneumonia). This is particularly true in the young animal, where immunological competence has not yet been fully achieved.

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ANAEMIAS ASSOCIATED WITH HELMINTHIC INFECTIONS

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Abstract

ANAEMIAS ASSOCIATED WITH HELMINTHIC INFECTIONS. This paper attempts to review the isotopic methods which have been utilized in the study of anaemias, particularly those associated with helminthic diseases. The main interest is in the detection and quantitation of gastrointestinal haemorrhage, and various isotopic methods currently employed in these measurements are discussed. The value of the various methods is acknowledged, but emphasis is made of the many inaccuracies which can be encountered, in particular the variations found between the various species. Although these factors do not invalidate the general results, it underlines the necessity of careful evaluation of the findings.

A brief review of the isotopic methods utilized in studying erythropoietic pathways is also given and it is hoped that it will serve as a basis for further discussions.

Many helminth infections are characterized by a concomitant anaemia which is directly attributable to the activities of the parasites. The parasites which can be classified as exerting their main pathogenic effect by haematophagia include the hookworms, *Necator americanus*, *Ancylostoma duodenale*, *A. caninum*, *Gaigeria pachyscelis*, *Bunostomum trigonocephalum* and *B. phlebotomum* and the stomach worms *Haemonchus contortus*, *H. placei*, and *Mecistocirrus digitatus*. Heavy infections with liver flukes, *Fasciola hepatica*, *F. gigantica*, *F. magna* and *Dicrocoelium dendriticum* are invariably accompanied by anaemia. Other parasites implicated in the production of anaemias are *Oesophagostomum columbianum* and *Chabertia ovina* in the sheep and the redworm infections of the genus Strongylus in the horse.

Infections of *Ostertagia ostertagi* in cattle have been associated with anaemia by some investigators [1] although other workers [2, 3] have reported insignificant changes in haematological indices; this is not inconsistent with the current findings that the type I disease produces no detectable anaemia while the type II disease is associated with a moderate anaemia of the normochromic, normocytic type [4, 5].

These anaemias can be caused by one or a combination of at least three factors: (i) a partial failure of erythropoiesis; (ii) an increase in intravascular destruction of formed red cells; and (iii) an abnormal loss of red cells due to haemorrhage. The last is perhaps the easiest to study, and it is this aspect of the anaemias of parasitic diseases that has been most intensively investigated, particularly with respect to hookworm disease, haemonchiasis and fascioliasis.

Anaemias due to haemorrhagic blood loss

In any anaemia where loss of blood into the alimentary tract is suspected, the best proof of its etiology is the quantitative measurement of this loss. If this volume of blood is then removed over the same period from a normal animal, it should then produce the same degree and type of anaemia. Care has to be exercised in phlebotomy experiments as it is not known how much reutilization of the lost blood takes place in, for example, the reabsorption of haemoglobin breakdown products, especially iron and amino acids, and it is possible to reach a stage when the anaemia is no longer a straightforward haemorrhagic anaemia but is partly due to a limitation of erythropoiesis because of a lack of essential precursors.

The first attempts at quantitation of the blood consumed by parasites was made in 1931 by Wells, who collected drops of blood from the anus of hookworms and counted the number of red cells in each drop [6]. By reference to the red cell count of the circulating blood he was able to estimate the amount of blood represented by each drop over a period of 22 minutes. Making the assumption that the rate of blood loss was constant throughout the 24-hour period, he then calculated the daily blood loss per worm as 0.84 ml blood per worm. Nishi (1933) repeated this work and concluded that 0.144 ml per 24 hours was a more realistic figure with a maximum of 0.392 ml per 24 hours [7].

A different approach to the problem was devised by Martin and Clunies-Ross [8] to estimate the minimal blood loss caused by *H. contortus* in sheep. They estimated the amount of phosphorus in *H. contortus* eggs and calculated the amount of blood that the parasites would have to consume to supply this amount of phosphorus.

These two approaches took no account of the blood consumed by the male parasites as in each case only female worms were involved in the estimations.

Andrews [9] attempted to measure the blood loss due to haemonchiasis using a modification of the benzidine technique to measure faecal blood and estimated that two animals lost approximately $1\frac{1}{2}$ to $2\frac{1}{2}$ times their total blood volume over a period of 10 days during a fatal infection of *H. contortus.*

The advent of techniques involving radioisotopes for the labelling of red blood cells provided a marker for the red cell. These techniques were primarily used for the study of circulating red cell volumes and initially red cells labelled with ^{32}P were used. The technique was developed from basic experiments of Hevesy and co-workers [10].

Isotopically-labelled cells (and plasma) were first used for the measurement of parasitic blood loss in *F. hepatica* infections of rabbits [11-13]. The ^{32}P-labelled cells and/or ^{131}I-labelled serum albumin were injected into fluke-infected rabbits and uninfected control rabbits, and after one hour the rabbits were killed and the radioactivity found in the flukes was compared with that in the circulating blood. Making the assumption that the feeding habits of the flukes were constant throughout the 24-hour period, a blood loss of 0.2 ml blood per fluke per 24 hours was calculated. The ^{32}P-labelled cells gave consistently higher results than the ^{131}I-labelled albumin.

Both ^{32}P-labelled cells and ^{131}I-labelled albumin have serious disadvantages in the estimation of blood loss. The ideal label is one which remains firmly attached to the red cell, and at the same time does not alter its potential life span. When the cell becomes effete and/or is destroyed, this label should not be reutilized but should be quantitatively excreted, preferably in the urine; if haemorrhage occurs into the alimentary tract, then the label must not be reabsorbed but should be quantitatively recovered in the faeces.

The red cell label most closely approximating to all these conditions is ^{51}Cr, first used for the estimation of circulating red cell volumes [14, 15] and then for red cell survival times [16].

Chromium-51-labelled red cells were first utilized to detect haemorrhage into the alimentary tract [17, 18] and then to obtain a quantitative measure of the blood loss due to hookworms in humans [19, 20] and in dogs [21 to 24].

It has been adequately demonstrated that the majority of the radioactivity is recovered in the faeces if ^{51}Cr-labelled cells are administered orally or injected directly into the alimentary tract [17, 20, 21]. However, at least in the sheep [25], a small percentage of the ^{51}Cr can be absorbed from the alimentary tract into the blood, although absorption can be reduced if cells labelled with inactive chromium are circulating. Apparently the presence of chromium in the blood inhibits the absorption of more chromium from the alimentary canal. This observation may only be pertinent in studies on ruminants as the percentage recovery of administered radioactivity in the sheep, even where the cells were labelled with inactive chromium, was only 88% (range 60 to 97%) [25] while recovery rates for humans and dogs were 96% (range 91 to 104%) [20] and 97% (range 93 to 100%) [21], respectively, without the presence of circulating inactive chromium.

When autologous ^{51}Cr-labelled red cells are injected into the bovine there is a rapid loss of activity over the first 18 to 24 hours to between 50 to 70% of the initial activity [26-28]. A similar rate of disappearance of labelled cells occurs in the sheep [29, 30]. After this phase is completed, there is a slower exponential loss of labelled cells but the slope of the disappearance curve for injected activity is much greater than that obtained with other species. This increased disappearance rate may be due to a shorter life span of bovine red cell and/or to a greater rate of elution of ^{51}Cr from the cell. Because of this, it has been suggested that ^{51}Cr-labelled red cells cannot be used for a reliable measurement of gastrointestinal blood loss in the bovine [26]. However, provided the elution rate of chromium from the red cell is constant in both normal and anaemic animals, the initial rapid loss of activity of ^{51}Cr from bovine red cells [26, 27] over the first 24 hours need not invalidate the subsequent estimation of faecal blood loss. The eluted ^{51}Cr is largely excreted in the urine and provided one allows sufficient time for the blood radioactivity to decline to the exponential part of the disappearance curve, then the radioactivity in the faeces can be related with reasonable accuracy to the blood activity. If, however, this eluted activity is excreted via the bile then an erroneously high estimate of gastrointestinal blood loss will result.

Variations in the elution of ^{51}Cr from red cells is dependent on differences in the haemoglobin molecules. In their original studies Gray and Sterling demonstrated that the globin portion of the molecule was the site of the ^{51}Cr attachment [14]. Later investigations have indicated that the binding sites were probably on the β chains of HbA $(\alpha_2\beta_2)$ [31 to 33] and there is one report that α chains can be labelled [34]. The elution of ^{51}Cr from these various haemoglobins has been studied in vitro and minor variants (single amino acid substitution) have little effect, HbS and HbC having a similar elution rate to HbA [33]. There are conflicting reports concerning elution of ^{51}Cr from HbF; dialysis experiments of labelled red cells have shown a similar [35, 36] or an increased [37] rate of elution as compared to labelled red cells of HbA. However, in vitro transfusion studies [38] have shown that the elution rates of chromium from HbF and HbC are not sufficiently different to merit the use of correction factors. The elution of chromium from HbC-S, however, is significantly greater, 3.5%/day compared to 1.8%/day for HbC and 0.85%/day for HbF, but the relatively short circulating half-life of HbC-S makes it unnecessary to apply correction factors.

This elution factor must be borne in mind in all circulating half-life studies, because in the anaemic animal a shortened red cell life may be a combination of an increased destruction of red cells and an increased elution rate. If the destruction of the red cells is due to haemorrhage alone, then an increased elution of the label will be reflected in increased urinary excretion; if, however, the destruction is haemolytic, then the two effects will be additive and the increased elution of the chromium label will give an overestimate of red cell destruction. This may be important in severe anaemias, as it has been shown that a new type of haemoglobin can be produced in sheep which have been made anaemic by phlebotomy [39]. This new haemoglobin HbAα was produced in sheep with HbA and it was postulated that it was a relatively unfinished haemoglobin which the immature erythrocytes produce after severe anaemic stress.

The overestimation of red cell destruction could be avoided with the use of an additional label for the circulating half-life studies, and of the alternative labels available the most promising is perhaps di-isopropylfluorphosphate (DF^{32}P). This label is very firmly attached to the cells and because of this always gives a higher value for circulating red cell life than ^{51}Cr-labelled red cells [40]. It also does not elute from sheep red cells [40] and it has been used simultaneously with ^{51}Cr-labelled red cells in the studies of *F. hepatica* infections of sheep [41].

Blood loss caused by gastrointestinal haemorrhage can also be measured indirectly, using labelled plasma proteins, especially if they can be used simultaneously with labelled red cells. The suitability of this plasma label is governed by the same conditions which are applied to the red cell label, viz., it should be firmly attached to the protein, it should not be reutilized to label other proteins, it should not be reabsorbed from the alimentary tract, and it should not alter the biological life of the protein. The isotopic labels of promise which fulfil one or more of these criteria are chromium, ^{51}Cr-labelled plasma albumin (chromic chloride), niobium, ^{95}Nb-labelled albumin, and copper, ^{67}Cu-labelled ceruloplasmin. All appear to be firmly attached to the protein although there is evidence that ^{51}Cr eluted or freed from albumin by catabolism may label other circulating proteins [42, 43]. If they are lost into the gut there is little reabsorption of all three, which makes them suitable for the detection of gastrointestinal loss of protein. However, both ^{95}Nb and ^{51}Cr have the disadvantage of altering the half-life of the labelled albumin compared to that measured by ^{131}I-labelled albumin [44]. Human serum albumin labelled with ^{51}Cr has been shown to have a biological half-life of seven-days [45]. Ceruloplasmin which has a normal half-life of approximately six days [46] is not altered by the ^{67}Cu labelling.

The big disadvantage in the use of labelled plasma proteins in measurement of gastrointestinal blood loss is that there is normally a substantial movement of plasma albumin into the gut. In normal humans this amounts each day to about 2g, equivalent to about 30 to 35 ml of serum, while red cell clearance to the gut as measured by the amount of ^{51}Cr recovered in the faeces was equivalent to about 1 ml of whole blood [45]. In normal sheep this loss amounted to approximately 7 ml of serum while red cell clearance was equivalent to about 2 ml of whole blood [47] and in rabbits it amounted to about 6 ml of serum and 0.1 ml of whole blood [47]. These obvious discrepancies make it impossible to measure whole-blood loss based on a loss of plasma proteins alone, because in a haemorrhagic anaemia it is possible that there is a disproportionate increase in this component of macromolecular leakage due to increases in gut permeability.

Iron-59-labelled haemoglobin has been utilized in studying the gastrointestinal blood loss caused by *Haemonchus contortus* in sheep [48-50]. These studies make the assumption that all the haemoglobin ^{59}Fe passing into the alimentary tract is lost in the faeces, a premise which has been shown to be false from simultaneous studies with ^{51}Cr and ^{59}Fe, even in normal sheep [25], and is likely to be even more erroneous in anaemic sheep where there are increased requirements for iron. There may, in these cases of parasitic blood loss, be a considerable alteration in the availability of the haemoglobin iron during its passage through the parasite.

The ^{59}Fe technique has the advantage that no corrections have to be made for elution of the label, as the normal breakdown of cells in the body releases the iron which is then incorporated into fresh haemoglobin. In addition, in normal animals extremely little iron is excreted in the faeces and therefore no corrections for normal excretion of the isotope need be applied in the calculations of blood loss due to the parasites.

Utilizing whole-body counting techniques [48] only small amounts of ^{59}Fe isotope are required (0.5 to 1.0 μCi in a lamb) and provided blood loss is moderate, the changes can be followed for considerable periods, giving a continuous assessment of blood loss. The use of whole-body counting procedures also means that the animals can be kept under natural conditions on pasture, as no elaborate collection of faeces and urine is necessary.

Anaemias due to failure of erythropoiesis

To study the failure of mechanism involved in erythropoiesis it is necessary to use labelled compounds which are incorporated directly into the haemoglobin molecule. The two most commonly used are ^{59}Fe and glycine-2-^{14}C, eight molecules of glycine being utilized in the formation of one molecule of protoporphyrin [51].

Iron can be utilized to study failures in iron absorption, and the rate at which it is incorporated into haemoglobin is indicative of the erythropoietic response. These ferrokinetic studies, combined with ^{51}Cr-labelled cells, have been used in the human to calculate a large number of parameters utilizing a series of formulae [52]. These include estimates of mean cell life [53], a red cell destruction index, a production index based on ^{51}Cr-labelled cells, a plasma iron turnover rate [54], and a red cell iron turnover rate. The latter two are based on ^{59}Fe studies. These indices are all related to the values obtained in normal human beings and it is this information with regard to the normal domestic animal which is largely missing and therefore these parameters have not yet been applied.

There are many problems inherent in the measurement of these indices. In the consideration of plasma iron turnover rate there are many variables, other than erythropoiesis, which influence its measurement, such as dilution of the isotope in enlarged iron stores, synthesis of other iron containing compounds, such as myoglobin and the iron containing enzymes and the early recycling of ^{59}Fe from rapidly destroyed erythrocytes. The last can be partially prevented by "blocking" with large doses of iron dextran a few hours after administration of the ^{59}Fe [51]. Plasma iron turnover rates can also be influenced by incorporation of ^{59}Fe into the molecule of haemoglobin after the sample has been taken but before the plasma has been separated from the red cells. This incorporation in vitro takes place in the reticulocytes [56-61]. However, provided there is cognizance of these limitations, then the information obtained from ferrokinetic studies will help to elucidate the part played by erythropoiesis in the etiology of anaemias.

In studies of erythrocyte survival times, glycine-2-^{14}C is the isotope of choice as, unlike ^{59}Fe, there is little reutilization of the label. It has been used in investigations in humans [62], swine [63], and sheep [64-66]. In the investigation of the Aoudad sheep, Cornelius et al. [65] contended that the particular animal investigated showed evidence of two cell populations, with different cell survival times. However, it has been pointed out that this situation could arise by shifts in haem production due to shifts in altitude and environment, as it takes more than a few weeks for haem production to stabilize [67].

In erythrocyte survival studies the technique of in vitro labelling, both with ^{59}Fe and glycine-2-^{14}C [56-61] of the immature erythrocytes is valuable as it ensures that there is not a prolonged labelling process with labelled cells appearing in the circulation over a considerable period. Unfortunately, there are reports that these immature cells may have a shorter survival time than normal cells, e.g. it has been shown in the rabbit that the life span of erythrocytes produced rapidly after acute haemorrhage is considerably reduced [68].

In investigations into erythropoiesis it is possible, using tritiated thymidine and the techniques of radioautography, to study the growth rates of haematopoietic cells [69]. Radioautographic techniques have been used to study the incorporation of both ^{59}Fe and ^{55}Fe into marrow cells and reticulocytes [70]. This type of investigation gives information on how the haemopoietic cells are responding, and if the anaemia is due to a failure of erythropoiesis, will give an indication at what stage this failure is taking place.

Finally, failure of erythropoiesis can be due to a failure of some extrinsic factor, such as iron, folic acid or vitamin B_{12}. The efficiency of iron absorption can be readily measured by administration of a small amount of ^{59}Fe and measuring the amount which passes out in the faeces. Even this simple technique has, however, some limitations, as it has been shown that the epithelial cells of the small intestine can take up iron, which is not passed into the circulation but is held. Eventually these cells are replaced by new cells and the activity appears in the faeces. This was first shown to happen in rats [71] and later confirmed in man [72]. In animals suffering from iron deficiency, the iron is rapidly absorbed and virtually none is retained in the epithelial cells. If an animal has an iron deficiency anaemia then care must be taken to evaluate cell survival studies as it has been shown that the red cells of iron deficiency anaemias have a reduced survival time [73, 74].

The value of radioactive isotopes in the study of all facets of anaemias is indisputable, and by a variation of suitable labels and techniques it is possible to screen practically all the stages in the life of a red cell. In the anaemias caused by parasites the main emphasis has been on the measurement of gastrointestinal blood loss, and there seems little doubt that in many conditions, especially haemonchiasis and fascioliasis, this is the major factor in the development of the anaemia. However, in some instances, e.g. fascioliasis, it would be advisable to investigate more fully the possibility of some impairment in erythropoiesis, both in the early migratory and in the chronic stages of the disease.

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USE OF LABELLED REFERENCE SUBSTANCES IN STUDIES ON INTESTINAL ABSORPTION AND DIGESTIBILITY

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Abstract

USE OF LABELLED REFERENCE SUBSTANCES IN STUDIES ON INTESTINAL ABSORPTION AND DIGESTIBILITY. There are several advantages in the use of reference substances over the classical balance technique, for studying the in vivo absorption of nutrients. Errors due to inaccuracies in estimation of feed intake and incomplete recovery of faeces are eliminated. Reference substances also permit the evaluation of short-term changes in absorption as well as the determination of the intestinal sites of absorption. A good reference substance should not be absorbed, should not modify the nutritional quality of the diet, and should be easily assayed. Most important, when absorption along the intestine is studied, the rate of passage of the reference substance along the intestine should be comparable to that of the nutrient under study. Labelled reference substances are preferable to non-labelled ones, as long as suitable facilities are available. This technique was found useful in the evaluation of calcium absorption in laying hens as a function of dietary calcium level and various physiological states.

It is suggested that this technique may be used to evaluate the degree of functional intestinal damage by parasitic infection, as well as the time course in the development of the disorder. Reference substances may also be used for accurate evaluation of changes in the rate of passage of nutrients through the digestive tract.

In studies of parasitic infections relatively little attention has been paid to the nutritional-physiological relationships between the host and parasite. By definition, the parasite lives at the expense of its host without contributing to the welfare of the latter, as in the case of symbiosis. Quite obviously, intestinal parasites, like ectoparasites, feed on materials taken from the animal, thus increasing its nutritional requirements. Moreover, intestinal parasites may damage the intestinal mucosa in such a way that the digestive and absorptive mechanisms are impaired. In general, such parasites may damage the intestinal surfaces, absorb the host's food, suck the host's blood and tissue fluids, cause mechanical obstruction of blood and lymphatic vessels, bile duct, or the alimentary canal itself, cause wounds resulting in haemorrhages and invasion of other pathogenic organisms, and secrete toxins and anti-enzymes.

From a physiological standpoint the parasites may reduce the absorptive capacity of the intestine directly by damaging the intestinal mucosa, or by increasing the passage rate of the nutrients through the intestine, leading in severe cases to diarrhoea.

Classically, absorption has been studied by the balance technique, which requires an accurate record of nutrient intake and faecal output. Let $V_{a\text{-net}}$ equal the net absorption.
Then $V_{a\text{-net}} = V_I - V_F$,
where V_I is the rate of nutrient intake and V_F is the corresponding faecal output.

Inherent in the balance method are difficulties in obtaining good recoveries of stool and accurate measurements of feed intake. These cause substantial errors which are largely cumulative and usually lead to erroneously high estimates of absorption. Furthermore, during parasite infection, it may be of interest to study the time course in the development of the intestinal disorder. For this purpose the balance method is completely unsuitable since it is usually designed on a relatively long-term basis.

Another common in-vivo approach involves measuring the disappearance of a single dose of labelled nutrient from the intestine. This technique, although useful for the purpose of diagnosis, does not measure the net absorption, as calculations are rarely based on specific activities of both intestinal contents and blood plasma. Furthermore, the absorption of an isotope, given in a soluble form, may differ from that of the stable nutrient if the latter is in poorly soluble form, as is often the case. Lastly, it is difficult, with this method, to account for the inflow of nutrient from the blood to the intestinal lumen.

Unabsorbable reference substances have been used for many years in the study of absorption. Unlabelled or labelled materials can be used. The former include chromic oxide, polyethylene glycol, and lignin [1]. The labelled reference substances include yttrium-91 [4, 5], cerium-144 [6], chromium-51 and scandium-47 [7], and lanthanum-140 [8].

To study absorption with the aid of a reference substance, the latter is mixed intimately with the feed and the ratio of nutrient/reference substance is determined in feed and faeces after a steady state of excretion has been reached. When it is desired to determine absorption from a given intestinal segment, these ratios are determined in food and the contents of the segments under study.

Net absorption is calculated from the expression:

$$\text{Per cent net absorption} = 100\,\left(1 - \frac{\text{S/R sample}}{\text{S/R diet}}\right),$$

where S/R is the ratio of the nutrient to the reference substance.

Compare this with the expression for the classical balance technique:

$$\text{Per cent net absorption} = 100\,\frac{S_i - S_F}{S_i},$$

where S_i = nutrient intake, and S_F = faecal output.

The advantages in using reference substances, compared with the classical balance, can be listed as follows: (a) The method is easier, since total faecal collection is not necessary, hence, the method is free from errors which arise from incomplete faecal collections and inaccuracies in intake measurements; (b) once steady state is reached, the method can be used to study variations in absorption with time, particularly short-term effects; (c) it can be used to follow the absorption pattern along the gastro-intestinal tract.

A good reference substance should meet the following specifications:

It should be unabsorbed

This is the most obvious requirement and may be easily tested by measuring the faecal recovery of an oral dose;

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[1] A review on such references was published by S. Hydin in: Digestive physiology and nutrition of the ruminant, LEWIS, D., (Ed.) Butterworths, London (1967).

It should move along the intestine at the same rate as the nutrient under study

This problem has not received sufficient attention, and considerable erroneous conclusions were based on studies in which these rates were not equal. For measurements of total intestinal absorption this problem is not so acute, since steady state results after a prolonged feeding, which permits accurate evaluation of absorption. Although the same argument may apply to absorption from an intestinal segment, in practice measurement depends on sampling the contents of the segment, and this may be misleading. Suppose, for example, that the reference substance is trapped in a segment but that this is not true for the nutrient. When the contents of the segment are then analysed they will contain a larger amount of the reference substance than actually flows through the segment at steady state, and net absorption will be overestimated.

The most obvious way to compare the passage rates is to administer both nutrient and reference substance in radioactive forms and measure their distribution along the intestine after various time intervals.

Inclusion of the reference substance should not alter the properties of the diet

Such as: reducing its nutrient density, or interact with nutrients.

It should be readily assayed

The use of labelled materials has two important advantages: they are added to the diet in minute amounts and therefore do not alter its properties; and radioassays are usually less tedious than chemical analyses. Against these advantages must be weighed the hazards involved in handling radioisotopes, complications that arise in mixing these compounds into the diet, and the experimental handling of the radioactive animals. Such work should not be attempted, therefore, by laboratories lacking suitable equipment and experience.

USE OF YTTRIUM-91 IN THE STUDY OF CALCIUM ABSORPTION IN THE LAYING FOWL

In the author's laboratory [5, 9] the absorption of calcium was studied with the aid of yttrium-91. Preliminary experiments indicated that this isotope was not absorbed by the laying hen, in agreement with results obtained with rats [4].

The rate of passage of yttrium-91 along the intestine was compared to that of calcium-45 by injection of the two isotopes into the upper duodenum and measurement of the isotopes' distribution in the intestinal contents at various intervals after dosing. The results presented in Fig. 1 indicate that both isotopes moved along the intestine at comparable rates.

A similar experiment, in which both isotopes were given per os, served to compare the rates of passage through the aesophagus and stomach. Results of this experiment suggest that the rate of emptying of calcium-45 from the stomach was considerably greater than that of yttrium-91. Marcus

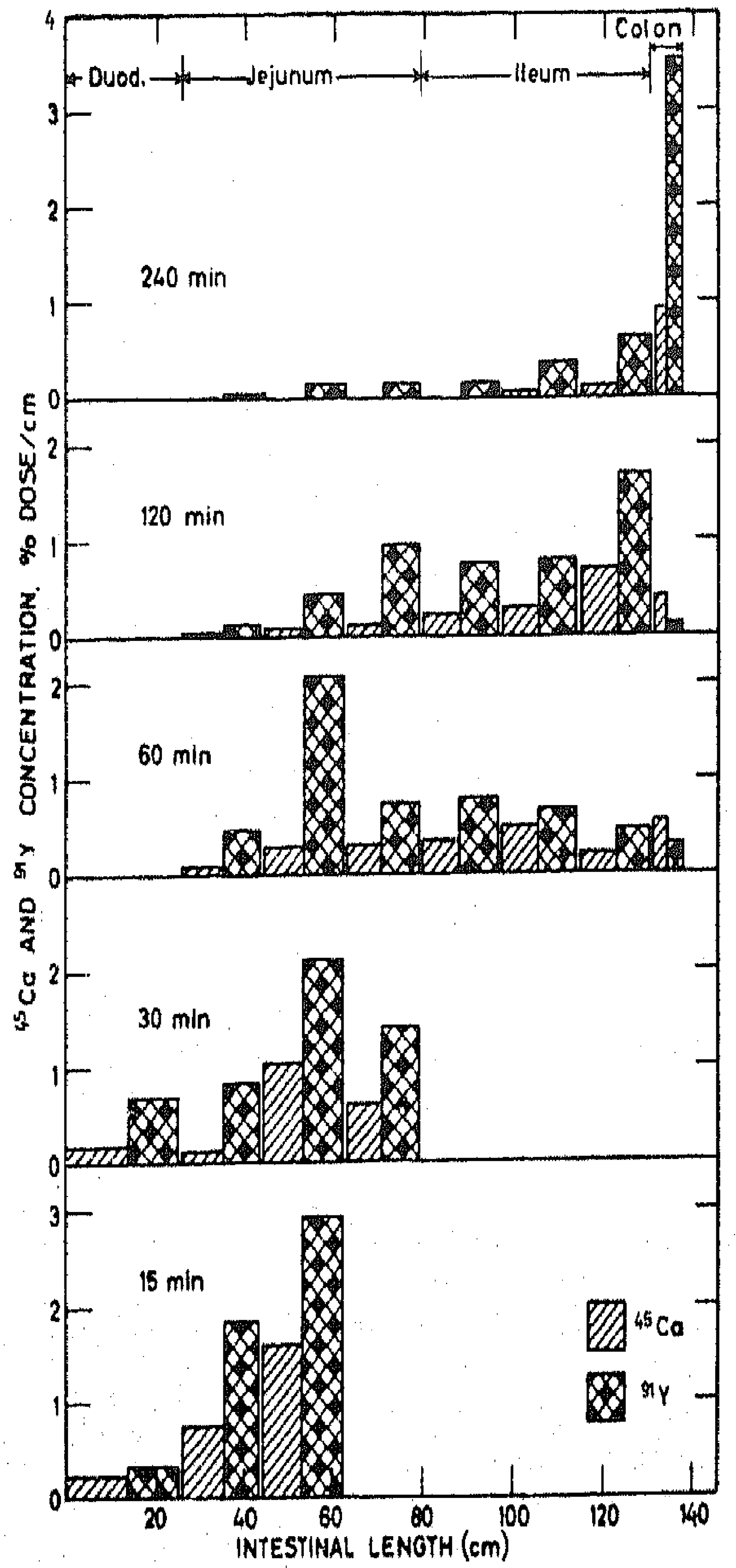

FIG.1. Amount of calcium-45 and yttrium-91 in the intestine of laying hens following the injection of a double tracer dose into the duodenum.

and Lengemann [4] have shown that, in their experiments, considerable amounts of yttrium-91 were adsorbed to solid particles from the intestinal digesta. Inasmuch as the crop and stomach of the laying hen are known to retain large particles longer than small particles and solutes, the adsorption of yttrium-91 on such particles may explain the longer passage time of this isotope. Since it takes the isotopes several hours to pass through the oesophagus and stomach, but may take only a few minutes in some parts of the small intestine, the author was unable to use the oral approach to estimate the rate of passage through the intestine. This was done by the duodenal approach, as discussed above.

On the basis of this evidence it may be concluded that yttrium-91 is not an ideal reference substance for calcium, since it does not have the same rate of passage through the stomach as calcium. However, once a steady state is reached, the calcium/yttrium-91 ratios in the intestine

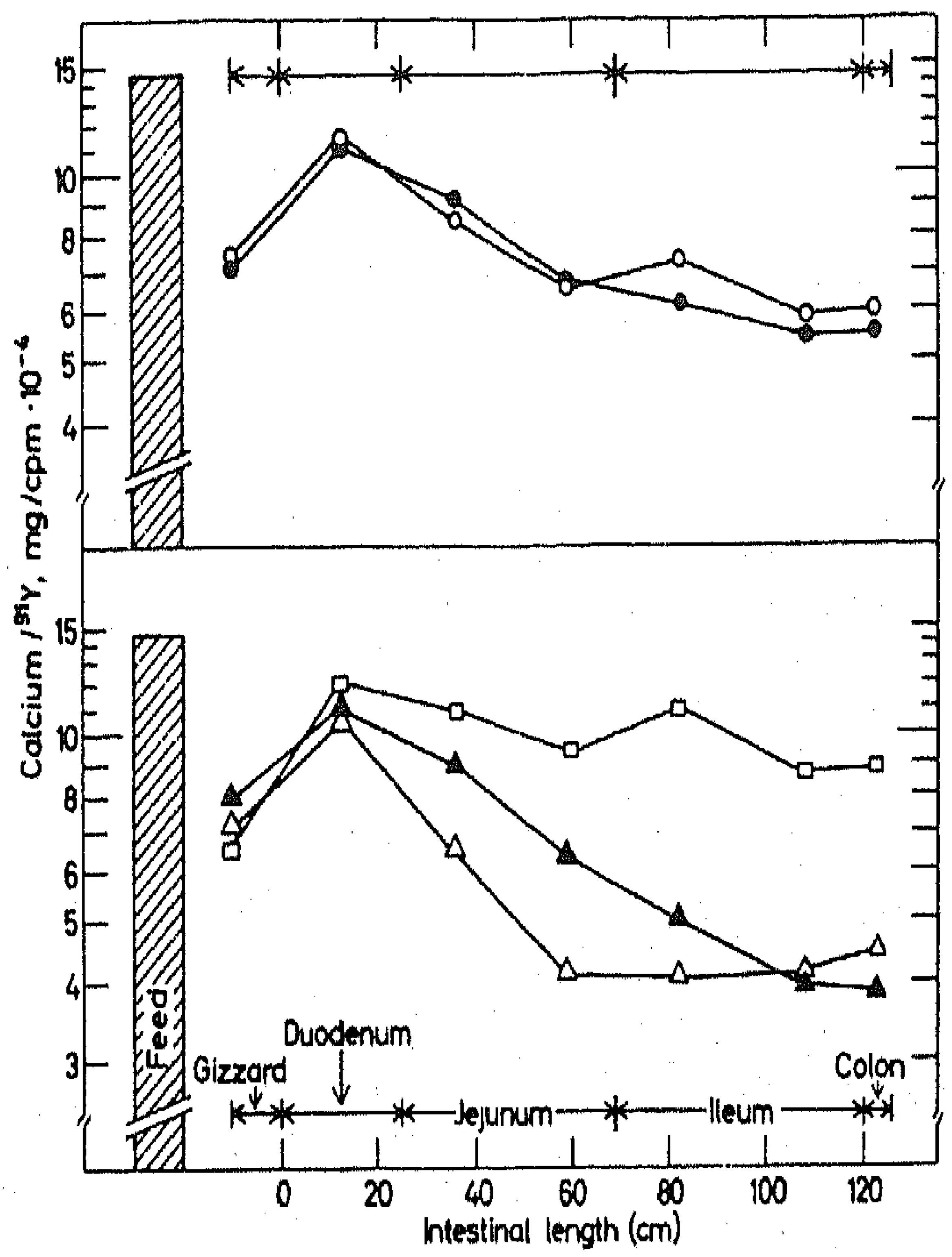

FIG.2. Calcium/yttrium-91 ratio in gastrointestinal segments of laying hens fed yttrium-91-labelled diets. Upper diagram: comparison between 1.90% calcium (●) and 3.56% calcium (○) diets. Lower diagram: comparison among periods of early egg shell calcification (△), late shell calcification (▲), and with no shell formation (□).

(excluding the stomach) can be used for estimating the net absorption since (a) it is not absorbed, (b) in the intestine it moves at a similar rate as calcium, (c) it is readily assayed, and (d) due to the minute amounts used it affects neither the nutritional nor physical properties of the diet.

Laying hens were fed diets containing yttrium-91 which had been initially thoroughly mixed with the dietary calcium carbonate and dicalcium-phosphate. The diets contained either 1.90 or 3.56% calcium. Four days later the birds were killed, during early shell formation, late shell formation, and when no shell was forming. Appropriate intestinal segments were ligated and their contents analysed for calcium and yttrium-91. Figure 2 shows the results in terms of Ca/Y ratios. It is evident that most of the calcium absorption occurred in the proximal intestine, with hardly any absorption in the lower ileum and colon. Dietary calcium had no effect on the percentage of net absorption. This can be interpreted to mean that the fraction of calcium absorbed was independent of the dietary calcium, at least at the levels studied. It is worth recalling that the minimum calcium requirement level for the laying hen is around 3% calcium. Figure 2 also shows that calcium absorption was doubled during periods of active shell deposition. This can be taken as evidence that the calcium absorption in laying hens is finely attuned to their demand for this mineral.

CONCLUSIONS

The experimental example presented here demonstrates the usefulness of a labelled reference substance for the estimation of absorption. By the use of such a substance the intestinal site of absorption was determined as well as the effect of dietary manipulations. Most important, this experiment demonstrates the usefulness of this technique in evaluating an almost hour-to-hour change in absorption.

It is suggested that the techniques described here may be used to determine the site of damage to the intestinal function by parasite infection. It is also possible to use reference substances to study the development of the disorder as well as the severity of the damage. The single administration of an unabsorbable reference substance may be used to estimate accurately the rate of passage of digesta along the intestinal tract, as influenced by the parasites.

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RECOMMENDATIONS

INTRODUCTION

Parasitic diseases represent serious problems to animal and man, particularly in the developing areas in the world. In view of the important role that ionizing radiation and radioisotopes play in the production and utilization of vaccines against such diseases, and of the probable importance of immunity and immunopathology in parasitic diseases, increased emphasis on research in this area is needed.

The Meeting takes cognizance, with pleasure, of the significant progress made in implementing some of the recommendations made by the Panel on the Production and Utilization of Radiation Vaccines against Helminthic Diseases, held in Vienna from 16 to 18 December 1963. Specifically, it is acknowledged that the recommendations to: hold meetings at regular intervals; make available research contracts for appropriate projects; provide training and exchange fellowships for scientists to visit laboratories actively engaged in irradiated vaccine research; retain from time to time a consultant responsible for summarizing the progress achieved in this general area; and maintain files of scientists working in the field of radiated vaccines, have been implemented. The Panel recommends that these measures be retained and extended as far as possible.

The Meeting also takes cognizance of the fact that basic knowledge of immunological phenomena in parasitic diseases is still lacking in many instances and that the acquisition of this information is essential to the useful application of radiation techniques and the study of parasitic diseases.

It is the opinion of the Meeting that the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, in close co-operation with other divisions of both organizations, and with other international organizations, such as the World Health Organization, can play an essential role and should take great interest in assisting workers in various nations to solve some of the unsolved problems. The World Health Organization and the Food and Agriculture Organization, through their technical services and expert panels, should propose leading subjects which at the present time are amenable for study by special techniques involving the use of radiation and isotopes.

Examples of areas in which further studies are required, and where progress can be anticipated, are:

(a) in vitro cultivation of infective and pathogenic forms of various parasites;

(b) studies on the metabolism of the parasite itself and on its host-parasite relationships, with special reference to nutritional aspects, immunological phenomena, pathophysiology, etc.

It is recommended that parasitologists be encouraged and aided to work in close co-operation with investigators in related disciplines (immunology, biochemistry, pathology, human and veterinary medicine, etc.). This could be achieved through regular meetings, through individual exchanges of research information, and by providing means by which research workers can spend some time in laboratories specializing in the above-mentioned allied disciplines.

In the interest of simplicity, the recommendations are given under three different headings:

A. Immunology
B. Pathogenic effects of parasites
C. Radiation-attenuated vaccines

They relate to the subject matter covered by the Meeting and are not meant to be comprehensive recommendations for the whole field of parasitology.

A. IMMUNOLOGY

Examples of investigations in the field of immunology which are urgent, require competent attention and are liable to result in significant accomplishment, include studies in the areas detailed below:

1. The immunopathological effects of parasitic infections include granuloma formation, pipestem fibrosis of the liver, and elephantiasis, as well as allergy and immediate hypersensitivity in nematode infections, anaemias in trypanosomiasis and malaria, malarial nephrosis, big spleen syndrome, etc. Radioactive isotopes offer useful tools in determining the extent of these effects. The reactions can be further studied by the immuno-suppressive effects of whole-body irradiation, immuno-suppressive drugs and anti-lymphocyte sera.

2. There is need to differentiate non-protective antigens, which may be useful in diagnosis, and protective antigens necessary for immunoprophylaxis. This could be accomplished by the use of radioisotopes in the development of highly sensitive serological tests, using isotope-labelled antisera and/or antigens, and fractionation techniques, such as chromatography and electrophoresis.

The latter could also aid in research for antigens common to parasites and their hosts, for host antigens modified by the parasite and of possible importance in immunopathology and in the characterization of anti-enzymes which may inhibit penetration and migration of parasites. Further studies with antigens might include the isotope tagging of parasites and/or parasitized erythrocytes to determine the mechanisms and rate of parasite destruction in immune and susceptible hosts.

3. By appropriate labelling the various types of antibodies detected by current serological tests should be differentiated into the various globulin classes with the hope that those that are effective in the protection against parasites and those which are responsible for immuno-pathological events might be identified, characterized and separated.

4. The course of antigenic development and release during the development of various parasites, and the antibody response to these, might most adequately be studied by radioisotope techniques. It is important to know whether apparent differences in antigens, and the antibody response to them, that may occur in different stages of life cycles are quantitative or qualitative differences, or both.

5. Progress should be hastened towards a clarification of the respective roles of humoral and cellular factors in parasitic infections. Techniques using radioactive compounds, such as thymidine and uridine, are now in general use for the analysis of the cellular aspects of immunology and these should be readily applicable to parasitic infections.

6. In view of the complex structure of many parasitic organisms (e.g. Fasciola hepatica, Schistosoma spp. Nematodes, etc.) there is need to determine the major sites of antigen production and antibody attachment within the parasitic organism. This might most readily be achieved by the use of autoradiographic studies at the light and electron microscopic levels, using radioisotope-labelled antisera, cells and metabolic products. In addition, dietary elements employed in the in vitro cultivation of parasites could be labelled to aid in the understanding of in vitro and in vivo nutritional requirements.

7. Marked proliferative responses frequently occur at epithelial and endothelial surfaces in the presence of various parasites (e.g. bile-duct epithelium in F. hepatica infection; abomasal mucosa in Ostertagia infection, arterial endothelium in Dirofilaria immitis infection, etc.). It is unknown whether this is the result of a direct action of parasitic materials or due to immunological mechanisms. It is recommended that studies be conducted on the effect of parasites and parasitic materials on cells using cell culture techniques and radioisotope-labelled precursors of DNA, RNA, etc.

8. The whole-body irradiated animal provides a convenient short-term host receptive to non-isogenic sera and cells: the use of such animals has the potential for detailed analysis of the several kinetic aspects of host-parasite interactions. Whole-body irradiation and other immunosuppressive techniques may also provide useful practical tools for extending the host range of parasites of medical and veterinary importance to readily available laboratory animals.

B. PATHOGENIC EFFECTS OF PARASITES

To assess precisely the pathogenic effects of parasites on their hosts we need to go beyond the question of disease incidence and mortality and to study the basic physiology of the host-parasite relationahip. Much useful information may be obtained by the application of conventional analytical biochemical methods, but it is completely impossible to study the kinetics of the systems involved without resorting to isotope techniques. Situations can exist where the concentrations of metabolites in body fluids of the host and even total pool sizes appear to be unaltered by the presence of parasites, but in fact the turnover in these pools is greatly increased. This is apparent from the work carried out so far on the protein metabolism of infected animals. It would seem particularly important that in the developing countries the facilities to carry out kinetic investigations should be available, otherwise the elucidation of local parasitic problems will be seriously delayed.

Particular attention should be paid to the hypoproteinaemias and anaemias which are so often associated with parasitic diseases. Isotopes have proved of considerable value in the investigations on these problems which have been carried out so far, but it is obvious that we are still far from a complete understanding of the mechanisms involved.

Significant leakages of plasma protein into the gastro-intestinal tract have now been demonstrated in a number of parasitic diseases but we still cannot assess the precise nutritional consequences of such effects to the host animal. More complete studies of gastro-intestinal functions of parasitized hosts are required.

It is important that changes in function should, as far as possible, be related to the structural changes which can be observed in the tissues of infected animals and, in this connection, it would seem that there is a great field for the application of radio-autographic techniques.

Closely associated with the functional changes in the parasitized host is the physiology of the worms themselves and in particular attention should be paid to how this is modified in the immune host.

The Meeting is making the following specific recommendations under heading B:

1. Anaemias

Isotopic techniques for studying blood-loss anaemias are relatively satisfactory. However, more work must be done to investigate those anaemias in which subnormal red cell production is more likely to be significant. This will involve studies on those processes associated with red cell production and destruction. For example: iron metabolism, copper metabolism, auto-immune phenomena, etc.

2. Hypoproteinaemias

Significant leakages of plasma protein into the gastro-intestinal tract in a number of parasitic diseases have been demonstrated. There is a need for the development of test substances labelled with short-lived isotopes which could be used for repeated determinations of gastro-intestinal leakage of macromolecules so that these measurements can be carried out at different stages of an infection and during diarrhoeaic and quiescent phases of the disease. More work must also be done on the alterations to digestive function brought about by gastro-intestinal parasites. It may be necessary to modify techniques used for normal animals so as to obtain valid measurements on a parasitized subject. For example, marker substances which are completely satisfactory in normal animals might give misleading results because of changes in permeability due to the presence of parasites.

Attention must be given not only to the nutritional but also to the immunological consequences of gastro-intestinal protein leakage; the increased degradation of immune globulins which occurs in the gastro-intestinal tract can bring about a significant impairment of protective mechanisms, particularly in the young animal which is immunologically immature. For example, puppies which are normally resistant to the "distemper complex" for a period of eight to nine weeks after birth, due to maternal antibody, lose their resistance much earlier when infected with hookworms.

There is an obvious association between the altered protein metabolism in the parasitized animal and the occurrence of anaemia; likewise, the existence of an anaemia will influence the protein metabolism.

Work so far has tended to be restricted to plasma albumin; there is a strong case to extend it to other plasma proteins. There has also been a tendency to concentrate on the degradative side of plasma protein metabolism. More attention should be given to protein synthesis and the manner in which it may be altered in parasitized animals.

3. Water and electrolyte metabolism

Attention should be given to the water and electrolytes (sodium, potassium, calcium, magnesium, phosphate, chloride, bicarbonate) metabolism of parasitized animals.

4. Parasite physiology

Little is known about the feeding habits, nutrition and physiology of many important parasites and the consequences of this to the host animal, especially when there may be competition for essential nutrients. A good example of such an effect is seen in Diphylobothrium infection in man, which causes an anaemia due to the uptake of Vitamin B_{12} by the worm, thus leading to a relative deprivation of the host.

5. Labelled drugs

More attention should be given to the use of isotopically-labelled antiparasitic drugs with a view to studying the uptake and metabolism by parasites and possibly their mode of action.

C. RADIATION ATTENUATED VACCINES[1]

REVIEW OF PRESENT STATUS

The present situation with regard to the use or development of vaccines against helminthic and protozoal diseases is summarized below.

Classification	Helminths	Protozoa and organisms of uncertain classification
In commercial use	Dictyocaulus viviparus Dictyocaulus filaria	-
Promising commercial application	Ancylostoma caninum Syngamus trachea	-
Encouraging laboratory results	Echinococcus granulosus	Plasmodium
Meriting further study	Schistosoma spp. Fasciola spp. Human hookworms Haemonchus and other gastro-intestinal nematodes	Trypanosoma spp. Anaplasma spp. Babesia spp. Theileria spp.

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[1] This term is used for vaccines prepared by radiation attenuation of parasites.

CONCLUSIONS AND RECOMMENDATIONS UNDER HEADING C

Helminths

Lungworms

The two lungworms (Dictyocaulus) vaccines are gaining increasing acceptance in agricultural practice.

Hookworms

An irradiated vaccine for hookworm disease of dogs is in an advanced state of development and is likely to be marketed in the near future. It is thought that consideration should be given to the development of human hookworm vaccines (Ancylostoma duodenale and Necator americanus) using apes as laboratory hosts. Because of its significance in human health, this should be considered in conjunction with other organizations (e.g. the World Health Organization).

Gapeworm (Syngamus trachea)

Laboratory experiments and preliminary field trials have given promising results. With respect to the problems of vaccination of the day-old chick, turkeys and game birds, and the technical problems of distribution of vaccine, it is suggested that vaccination in the hatchery would offer a practical solution. It is recommended that this work should be encouraged and continued.

Echinococcus

In view of the zoonotic significance in many countries (e.g. the Mediterranean littoral, Australia, New Zealand) and the results of preliminary experiments in which up to 92% protection has been achieved in dogs following vaccination with irradiated protoscolices, it is recommended that this work also be encouraged and continued.

Schistosomes

A considerable volume of knowledge has been accumulated from experimental infection of various laboratory hosts with irradiated Schistosoma mansoni and S. japonicum, and although the results do not currently justify application of this technique to the field of human vaccination, further research on this group of parasites should be encouraged with a view to investigating basic immunologic phenomena.

Liver fluke (Fasciola spp.)

Because of the widespread distribution and economic significance of these parasites, the possibility of vaccination using irradiation should continue to be explored as part of a general co-ordinated programme.

Haemonchus and other gastro-intestinal nematodes

The major problem in the development of a satisfactory vaccine to Haemonchus contortus appears to be a prolonged unresponsive period in lambs. This is of considerable immunological interest and irradiated vaccines may be of use as a tool in this study.

Protozoa

Malaria (Plasmodium)

The preliminary results in mice are encouraging and further expanded study is recommended.

Trypanosoma, Anaplasma, Babesia

Irradiation studies should be encouraged in view of their potential value in the development of vaccines and as an immunologic tool.

Theileria spp.

East Coast Fever is a serious problem in Central and East Africa. Neither chemotherapy nor vaccination is available and prophylaxis depends entirely on vector control. X-irradiation vaccines should be considered in this field.

COMMENTS AND RECOMMENDATIONS ON TECHNICAL AND THEORETICAL ASPECTS OF IRRADIATION

1. Further comparison of various ionizing radiations should be considered (e.g. X-rays, gamma rays and fast neutrons).
2. Reporting of results should take cognizance of new information on physical constants during irradiation (e.g. temperature of medium before and during irradiation, oxygen tension, etc.); see also recommendations of the IAEA Panel held in 1963[2].
3. The observed increase in infectivity of nematode larvae after exposure to low doses of radiation should be investigated as a biological phenomenon.
4. The basic mechanisms of radiation damage in helminths and the apparently superior immunogenic efficacy of irradiated larvae should be investigated.
5. Techniques, such as in-vitro cultivation and biochemical tests, might be considered as a method of assessing radiation attenuation.
6. Studies in long-term storage of helminth larvae and protozoa might facilitate the development of X-irradiation vaccines.

Whole-body irradiation

This may prove to be a useful procedure in studies on immunity to helminthic and protozoal infection.

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[2] Production and Utilization of Radiation Vaccines against Helminthic Diseases, TRS No. 30, IAEA, Vienna (1964).

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